AN ABSTRACT OF THE THESIS OF

Radhika Vidyasanker for the degree of Master of Science in Microbiology presented on December 13, 1999. Title: THE *RPSL* GENE AND STREPTOMYCIN RESISTANCE IN *STREPTOCOCCUS GORDONII* AND *STREPTOCOCCUS PYOGENES.*

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Abstract approved:

Dennis E. Hruby

Streptomycin resistance in both gram-positive and gram-negative bacteria is usually caused by a single mutation in the *rpsL* gene. The *rpsL* gene encodes the S12 protein of the ribosomal complex. The *rpsL* genes of various bacteria have consensus regions in their sequences. Primers were designed from these consensus pockets and a fragment of the *rpsL* gene was sequenced from *S. gordonii* using PCR based methodologies. Using the Multiplex Restriction Sequence PCR(mRS PCR), which used the known primer at one end and a restriction site primer on the other, a gene walk was conducted. In streptomycin resistant strains of *S. gordonii*, namely GP204, SP204 and SP635, the AAA coding for Lys56 was mutated to ACA, coding for Thr56. The lysine to

threonine transition, causing resistance to streptomycin was identical to that expected from the literature.

The streptomycin resistance gene of *S. pyogenes* was mapped using similar techniques. Streptomycin resistant strains S43 ATCC, S43/192/4 and S43/192/30R were studied. In streptomycin resistant S43 ATCC and S43/192/30R strains, the lysine 56 changed to isoleucine and threonine respectively. Surprisingly, the 192/4 had two mutations, in each of the two hotspots in the rpsL gene where mutations due to streptomycin resistance occur. It had the amino acid 56, lysine, mutated to arginine and lysine 101 changed to asparagine. To check if this mutation was stable in the host animal, S43/192/4 P8 (S43/192/4 passaged eight times in mice) was sequenced and the sequence was identical to the streptomycin resistant 192/4. Hence, the lys101 mutation was stable and unlike the ancillary mutations in *E.coli* and *S. typhimurium*, which are compensated by new mutations.

The pathogenesis of *S. pyogenes* depends in part on the ability of the pathogen to adhere to the epithelial cells of the throat and the quantity of M protein. Pathogenesis studies done on mice revealed the avirulence of S43/192/4^{SmR} strain. To elucidate the reason for this avirulence, the adherence properties and the production of M protein of the

two strains S43/192/4^{SmR} and S43/192/30R were tested. Qualitative immunoblot analysis of the M protein of 192/4 and 30R revealed no significant difference. Competition ELISA was conducted to quantitate the M protein, and this also did not show any significant difference in the M protein levels. The adherence of 30R and 192/4 was measured on human pharyngeal epithelial cell line. The adherence properties of S43/192/4 SmR, was no different from other strains in this experiment. Electron microscopy, using immunogold to highlight the M protein on the cell surface showed no differences.

THE *RPSL* GENE AND STREPTOMYCIN RESISTANCE IN *STREPTOCOCCUS GORDONII* AND *STREPTOCOCCUS PYOGENES*

by

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APPROVED

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Major Professor, representing Microbiology

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Chair of Department of Microbiology

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CHAPTER 1
THESIS INTRODUCTION

Streptococcal disease and the need for a vaccine

In 1920, Homer Smith reported, " As rheumatic fever is not generally reportable, it is impossible to obtain accurate statistics as to its incidence. It is very widespread in its occurrence and is found in practically all countries…" Today rheumatic fever is still the leading cause of heart disease in developing countries, particularly in school-age children and continues to cause disease sporadically in the developed world. *Streptococcus pyogenes* causes both the common "strep throat" (pharyngitis) and the sequelae to pharyngitis including rheumatic fever. The spectrum of diseases caused by *S. pyogenes* range from mild to life-threatening. Of particular importance is that in recent years, there have been reports of highly invasive

strains of *S. pyogenes* that cause necrotizing fasciitis. This pathology has given *Streptococcus* the label in popular press as the "flesh eating bacterium". Group A streptococcus also causes scarlet fever, streptococcal toxic shock syndrome, erysipelas, pyoderma, acute rheumatic fever, glomerulonephritis and a variety of other diseases (Fischetti et al., 1997).

Looking back at the history of streptococcal research, it took more than a quarter of a century to determine unequivocally that group A streptococcus causes rheumatic fever. Through the detailed research of Rebecca Lancefield, the pathogenic streptococci with their complex antigenic structure were grouped, which served as the basis for much future research. The virulence of group A streptococcus is determined by a variety of structural molecules, toxins and enzymes. Of primary importance are the hyaluronic acid capsule, exotoxins, streptolysins, streptokinases and perhaps most importantly, the M protein. The surface-located M protein, a α-helical coiled-coil fibrillar molecule, confers to the organism the ability to resist phagocytic attack (Fischetti et al., 1989). The serotypes of the M

protein vary tremendously with more than 200 currently recognized by the Center for Disease Control (CDC). Though streptococcal disease is caused by a large number of serotypes, M3 and M18 strains are the most common in the U.S, causing rheumatic fever and M1, M3 and M18 responsible for most invasive disease. The pathogen is spread by person-to-person contact via respiratory droplets. Transient colonization of the throat also occurs. It is estimated that 15 to 20% of the normal population are carriers for the Group A streptococcus (Murray et al., 1994).

Although the importance and need for a vaccine against *S. pyogenes* is well documented, as the antigens of *S. pyogenes* vary widely with serotypes, development of a successful vaccine has proved elusive. There was also some complacency within the medical research community because the group A streptococcus continues to be relatively sensitive to penicillin. Hence, the development of a vaccine was not a priority in many developed countries. However, with the appearance of enterococci resistant to penicillin-based antibiotics, the stage is set for the group A streptococcus to acquire this characteristic, with potentially disastrous

consequences. Furthermore, the reticence of medical practitioners to prescribe antibiotics for "routine" sore throats may lead to increased post streptococcal sequelae, such as rheumatic fever. It thus becomes imperative that streptococcal infections are prevented; an effective vaccine needs to be developed against *Streptococcus pyogenes*.

History of vaccines

Vaccination originated just over 200 years ago when Dr.Edward Jenner experimentally tested the folklore-based notion that human infection with a mild bovine pathogen called cowpox would protect infected individuals against human smallpox. Thus was discovered the principle of vaccination. Vaccination protects the recipient from pathogenic agents by establishing an immunological resistance to infection. An injected or oral vaccine induces the host to generate antibodies against the pathogen; therefore, during future exposures the ability of the pathogen to cause disease is limited by the immune system (Janeway et al., 1997).

Modern vaccines typically consist of either a killed or a live, nonvirulent (attenuated) form of the infectious agent. Traditionally, the infectious agent is grown in culture, purified, and either inactivated or attenuated without losing the ability to evoke an immune response that is effective against the virulent infectious organism.

Although this procedure has demonstrated success against many diseases, there are limitations.

1. Not all-infectious agents can be grown in culture; hence, no vaccines have been developed for a number of diseases, such as *Mycobacterium leprae*.
2. Complete immunity is not developed by the body when vaccines are injected. Induction of local immune responses characterized by IgA, requires mucosal presentation.
3. Attenuated strains may revert, as was seen with polio vaccine in Germany in the early phases of the mass vaccination program during the 1950s.
4. Inactivation may be incomplete and the handling of large volumes of the pathogen creates many safety hazards.

Recombinant DNA technology has provided a means of creating a new generation of vaccines that overcome the drawbacks of traditional vaccines. Virulence genes could be deleted from an infectious agent that retains the ability to stimulate an immunological response. This is theoretically safe, as it is impossible to acquire a whole set of genes.

Bacterial commensal vectors

To circumvent some of the safety and environmental issues inherent in the wide scale dissemination of engineered pathogens, non- pathogenic vectors have been developed. This delivery system is referred to as the bacterial commensal vaccine. Live nonpathogenic carrier systems that encode discrete antigenic determinants of a pathogenic agent can be created. In this form, the carrier system facilitates the induction of a strong immunological response against the pathogen. Non-pathogenic gram-positive vectors were developed which stimulate a mucosal immune response at the site at which

the pathogen enters the mammalian host, with the ultimate aim of blocking infection at this site (Pozzi et al., 1992b), (Fischetti et al., 1993). The system enables the display of foreign antigens on the bacterial surface (Medaglini et al., 1995). Thus, gram-positive oral commensal bacteria expressing recombinant fusion proteins on their cell surface have been successfully used to raise both a mucosal and a systemic immune response to foreign antigens while colonizing the oropharynx, respiratory tract or vaginal mucosa (Bessen et al., 1990)(Fischetti et al., 1993).

Several approaches are being explored to produce an effective vaccine against *S. pyogenes*. Although the antigens of this bacterium vary considerably, there are certain epitopes within the M protein, which are highly conserved. These epitopes are the immunogenic component of at least one prototype vaccine. Other methods are the vaccines targeting different parts such as the surface molecule C5a peptidase, the group carbohydrate, or the secreted streptococcal protease (*Spe B*).

The M protein is immunogenic and the formation of antibodies against M protein is essential to limit infection. Disease occurs when strains establish an

infection in the pharynx before specific systemic antibodies develop. When designing a vaccine, it is important to remember that an IgA inducing mucosal vaccine should be the most effective vaccine. When *S. pyogenes* colonizes the oropharynx, the IgA of the mucosa would prevent the disease whilst the IgG in the body would not be exposed to the pathogen until the disease has progressed and the pathogen is disseminating through the blood stream (McGhee et al., 1993).

An important criterion in vaccine design was to choose the vaccine design. Commensal vaccines, as discussed earlier, remove many of the inherent dangers associated with using the pathogen. With *S. pyogenes* vaccine, *Streptococcus gordonii* was an attractive choice. *S. gordonii*, formerly known as *S. sanguis* belongs to Lancefield's group H. It is also commonly referred to as the "Challis" strain. *S. gordonii* is a commensal bacterium isolated from the human oral cavity and was found to be naturally competent for genetic transformation (Pozzi et al., 1987) (Pozzi et al., 1990).

A recombinant *S. gordonii* was constructed which expresses the conserved C repeat region of *S. pyogenes* M

protein. A pSMB104 plasmid construct was made: pSMB104 *emm-6.1*$_{\Delta 104}$, a gene encoding for the 245 amino acid M6$_{\Delta 104}$ protein containing the first 16 N-terminal and the last 220 C-terminal amino acids of M6 (Bessen et al, 1990), (Oggioni et al., 1996). This construction lacks the serotypically diverse immunodeterminant epitopes that have thwarted previous vaccine development efforts. This plasmid was transformed into *S. gordonii* Challis where the *emm-6.1*$_{\Delta 104}$ gene integrated into the bacterial chromosome (Bessen et al., 1989) (Bessen et al., 1995). Thus, the recombinant S.gordonii expressed the C repeat region on the bacterial surface, inducing IgA and IgG when introduced into animals (Myscofski et al., 1998) (Medaglini et al., 1995) (Fischetti et al., 1993) (Fischetti et al., 1996) (Fischetti et al., 1997).

Any live vaccine candidate needs a marker to identify the vaccine strain and to differentiate the vaccine strain from the naturally occurring commensal strain, both during growth in the laboratory and in the immunized target species. The important attribute of a marker is to easily identify and differentiate the vaccine strain from other strains. Antibiotic markers

have been markers of choice as the antibiotic resistance is easily identifiable on the basis of growth of a colony on a plate. Care must be exercised to see that this antibiotic resistance is not transferable to the other bacteria in the environment and hence a plasmid marker is not advisable. Care should also be taken to see that the antibiotic used does not give any advantage to the vaccine strains.

Streptomycin resistance was chosen as the antibiotic resistance marker in *S. gordonii*, because streptomycin is neither used prophylactically in any streptococcal infection, nor commonly prescribed in general. Further in the literature studies, most streptomycin resistance in both gram-negative and gram-positive bacteria is shown to be caused by a single mutation in the *rpsL* gene (Salles et al., 1993). Thus as a chromosomal mutation caused the resistance, it was not readily transferable.

Streptomycin Resistance

Waksman discovered streptomycin in 1943. Since then, several aminoglycosides have been added to the impressive array of antibiotics. The mode of action of aminoglycosides is that they bind irreversibly to the 30S bacterial ribosome. This binding interferes with the translation of the genetic code, and inhibits synthesis of protein. An essential prelude to ribosomal binding is an energy and oxygen-dependant transport mechanism that is inhibited by anaerobiosis and low pH. The uptake of aminoglycosides for various organisms, specifically gram-positive cocci is facilitated by the presence of inhibitors of synthesis of the bacterial cell wall - that is, β lactam antibiotics and vancomycin.

Microbial resistance against aminoglycosides is mediated through the following mechanisms: a plasmid-mediated aminoglycoside-modifying enzymes, reduced transport into the cell and a ribosomal mutation that results in reduced affinity for the 30S subunit. The last mentioned mechanism is primarily demonstrated for streptomycin (Mayo Clin Proc, 1999).

In most bacteria, point mutations which cause streptomycin resistance occur in the *rpsL* gene. The *rpsL* gene encodes the conserved rps12 or the S12 protein of the ribosomal accuracy center (Tolvonen et al., 1999). The ribosome accuracy center is a highly conserved component of the cellular apparatus of translation. This comprises a ribosomal RNA domain (16S RNA) and several polypeptides of the small subunit including S12, S4, ands S5 (*E.coli* nomenclature)(Alksne et al., 1993). As discussed earlier, streptomycin causes a decrease in the translational fidelity of the ribosome (Garvin et al., 1974), probably by inhibiting its proof-reading mechanism. Mutations in S12 protein alleviate this effect. These mutations reduce the affinity of the ribosome for streptomycin and result in a slower rate of translation, with a concomitant increase in translational accuracy (Galas and Branscomb, 1976). Mutations that occur in S4 and S5 proteins, called ribosomal ambiguity mutations (*ram*), cause an opposite effect, increasing the affinity for streptomycin (Bock et al., 1979) and causing a relaxation in the translational fidelity of the ribosome (Andersson and Kurland, 1983).

Stern et al (1988) identified the sites of interaction between 16S RNA and S12 protein; interestingly, this region is adjacent to the putative site for streptomycin interaction. Hence, mutations in S12 that promote streptomycin resistance may protect the streptomycin-binding site in the RNA (Timms et al., 1993). In streptomycin resistant mutants, the rate of formation and the initial complex between a near cognate tRNA and the ribosome was decreased. The proof-reading control, as assessed by the amount of GTP consumed per erroneous incorporation, was increased with ribosomes from streptomycin resistant mutants. The association between subunits was loosened in ribosomes, since they dissociate more readily than wild type ribosomes upon heating (Phoenix et al., 1983). Gorini showed that ribosomal protein mutations conferring resistance to streptomycin in *E.coli* sometimes caused hyperaccurate translation (Rondanelli et al., 1964). These mutations were found frequently in the ribosomal protein S12 and occurred on specific residues. Later work established that mutations in proteins S4 and S5 caused increased sensitivity to streptomycin and a loss of translational accuracy.

THE *RPSL* GENE AND STREPTOMYCIN RESISTANCE IN COMMENSAL BACTERIUM *STREPTOCOCCUS. GORDONII*

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Introduction

A traditional method of identifying a bacterial gene function is to transform bacteria that lack this function (i.e., a mutant), with plasmids containing a library of small fragments of the chromosomal DNA from a wild type bacterium. If the DNA fragment in the plasmid complements the missing function, the transformed bacteria will grow as wild type under selection. The rescuing fragment can then be sequenced and the nature of the encoded gene product deduced by the use of bioinformatics (Sambrook et al., 1989). Another method has recently emerged for identifying genes that are predicted to have conserved sequences. Complementary primers are designed from these conserved regions and a portion of the gene is excised and sequenced using the polymerase chain reaction (PCR). "Gene walking" is then used to pull out the whole gene. The advantages of this method are that it is quick and easy to perform. The limitations of this method are the availability of conserved sequences for the gene of interest and good techniques in PCR and gene walking. Streptomycin

sensitivity is dominant and hence the gene encoding streptomycin resistance might be difficult to identify in the library technique. Both the library method and the PCR technique have worked in identifying the *rpsL* gene in various bacteria (Salles et al 1992; Nair et al 1993). Most of the streptomycin resistance in gram positive and gram-negative bacteria is caused by a mutation in the *rpsL* gene. By comparing the *rpsL* gene from various bacteria, it is evident that there are conserved regions in the gene, as shown in Figure 2.1. Not surprisingly, just as certain parts of the gene are conserved, the mutations that occur in this gene to confer resistance to streptomycin also seem to be restricted to two regions. Phenotypically, streptomycin-resistant mutants can be divided into two classes, those resistant to streptomycin but able to grow in its absence (Sm^r) and those whose growth depends on continued presence of streptomycin (Sm^d). Both classes of mutants have alterations in the *rpsL* gene. Since most of the mutations causing streptomycin resistance occurred in the *rpsL* gene, (Salles et al 1992; Kenney et al 1994; Bjorkman et al 1999) it seemed logical that to decipher the streptomycin

Figure 2 1. Similarities in the S12 protein from various bacteria. This alignment was done using *omiga* software. Each amino acid is represented in a different colored box. This facilitates comparison of amino acid sequences from all the bacteria. Several pockets of consensus sequences are evident, for example, amino acids 1 to 20 and 41 to 120. These are the consensus sequences targeted for designing primers as explained in the text. Amino acid 1 starts with the amino terminal and this figure is based on the single amino acid code. The bacteria listed (in the order of the tabulation) are, *Borrelia burgdorferri, Helicobacter pylori, Micrococcus luteus, Mycobacterium tuberculosis, Staphylococcus aureus, Streptococcus pneumoniae* and *Salmonella typhimurium*.

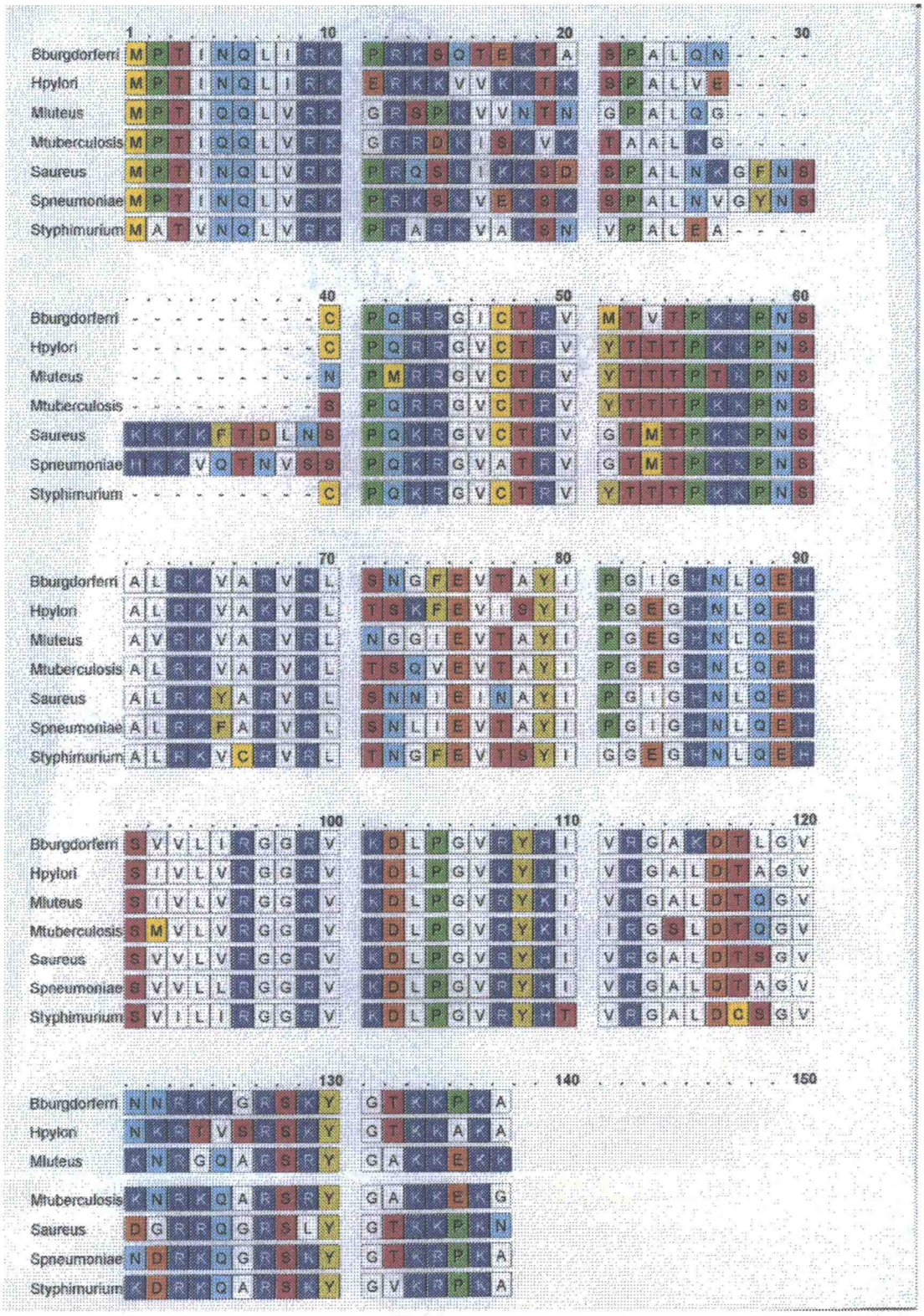

Bburgdorferi
Hpylori
Mluteus
Mtuberculosis
Saureus
Spneumoniae
Styphimurium

Figure 2.1.

resistance in S. gordonii, the S12 gene in S. gordonii should be examined first.

Materials and Methods

Strains of *S. gordonii*

The strains of *S. gordonii* chosen for this study should help elucidate the differences in streptomycin resistant and streptomycin sensitive strains (Figure 2.2). The parental *S. gordonii* Challis strain, V288 is a well-studied strain, which is streptomycin sensitive. The Challis strain was made streptomycin resistant in the laboratory, using methods described by Sambrook (1989). The resistant strain is called GP204. Two other strains were also used in this study, SP204 (1-1) and SP635. Both these strains are streptomycin resistant *S. gordonii*. SP204 (1-1) also is thymidine kinase deficient by virtue of a point mutation in the thymidine kinase gene. The GP204 strain was exposed to mutational techniques and this point mutation was selected with FudR

in the laboratory (Chris Franke, SIGA Pharmaceuticals). This strain was studied to confirm that the mutation causing streptomycin resistance was stable, and not affected by laboratory passages on the bench.This strain was also derived from V288. The strain SP635 contains the C repeat region of *S. pyogenes* integrated behind a promoter in the *S. gordonii* chromosome. These strains are detailed further in Figure 2.2.

PCR

Primers Used

The primers used in the PCR reaction, CF56, CF57 and CF58 were designed from the conserved regions of the *rpsL* gene. The *rpsL* genes from various bacteria were compared and the conserved regions identified. The direction of primer CF56 is downstream. The primers CF57 and CF58 are primers going upstream. These primers contain degenerate bases at several positions. The design of these primers is indicated in Figure 2.3.

Figure 2.2. The strains of *S. gordonii* used in this study. The strain V288 is the ``Challis'' strain of S. gordonii, which is the wild type streptomycin sensitive strain. The rest are laboratory strains derived from V288, with various added features, as indicated.

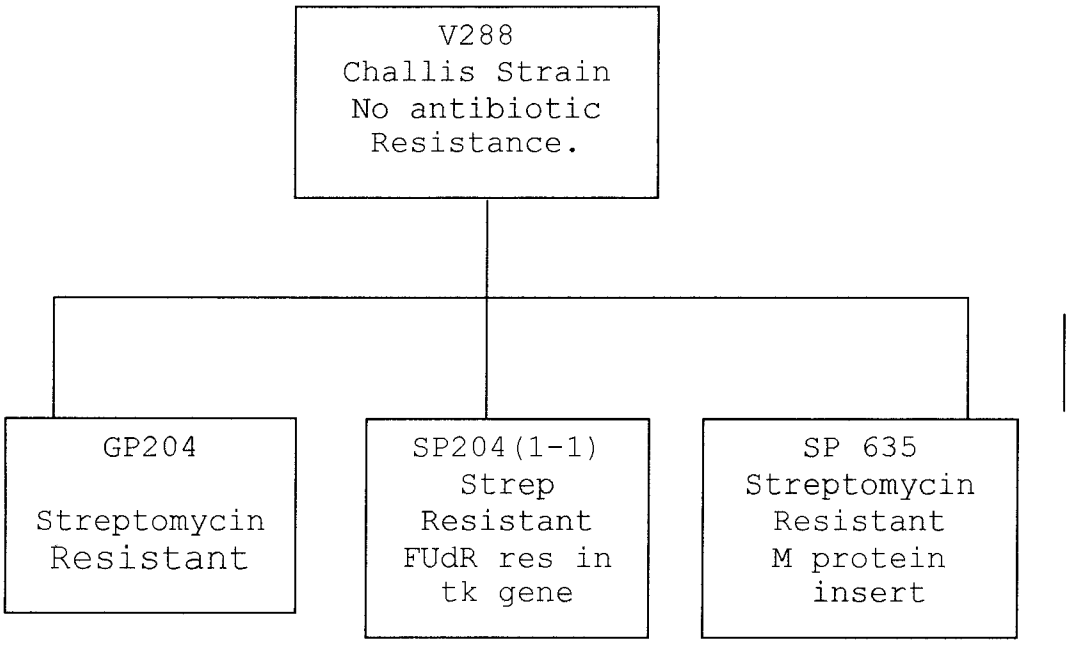

V288
Challis Strain
No antibiotic
Resistance.
GP204
Streptomycin
Resistant
SP204(1-1)
Strep
Resistant
FUdR res in
tk gene
SP 635
Streptomycin
Resistant
M protein
insert

Figure 2.2.

Figure 2.3. Designing of primers CF56, CF57 and CF58. The nucleotide sequences of the S12 gene of *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus pneumoniae* shown in the three pages. The sequences align very well. The consensus sequence is shown with the location of the 3 primers situated in the consensus sequence. The primer from 170 to 190 in the 5' strand is the primer CF56. The primers CF57 and CF58 overlap and are situated in the 3' strand. The primer from base pair 361 to 380 is CF58 and the primer from 366 to 387 is CF57.

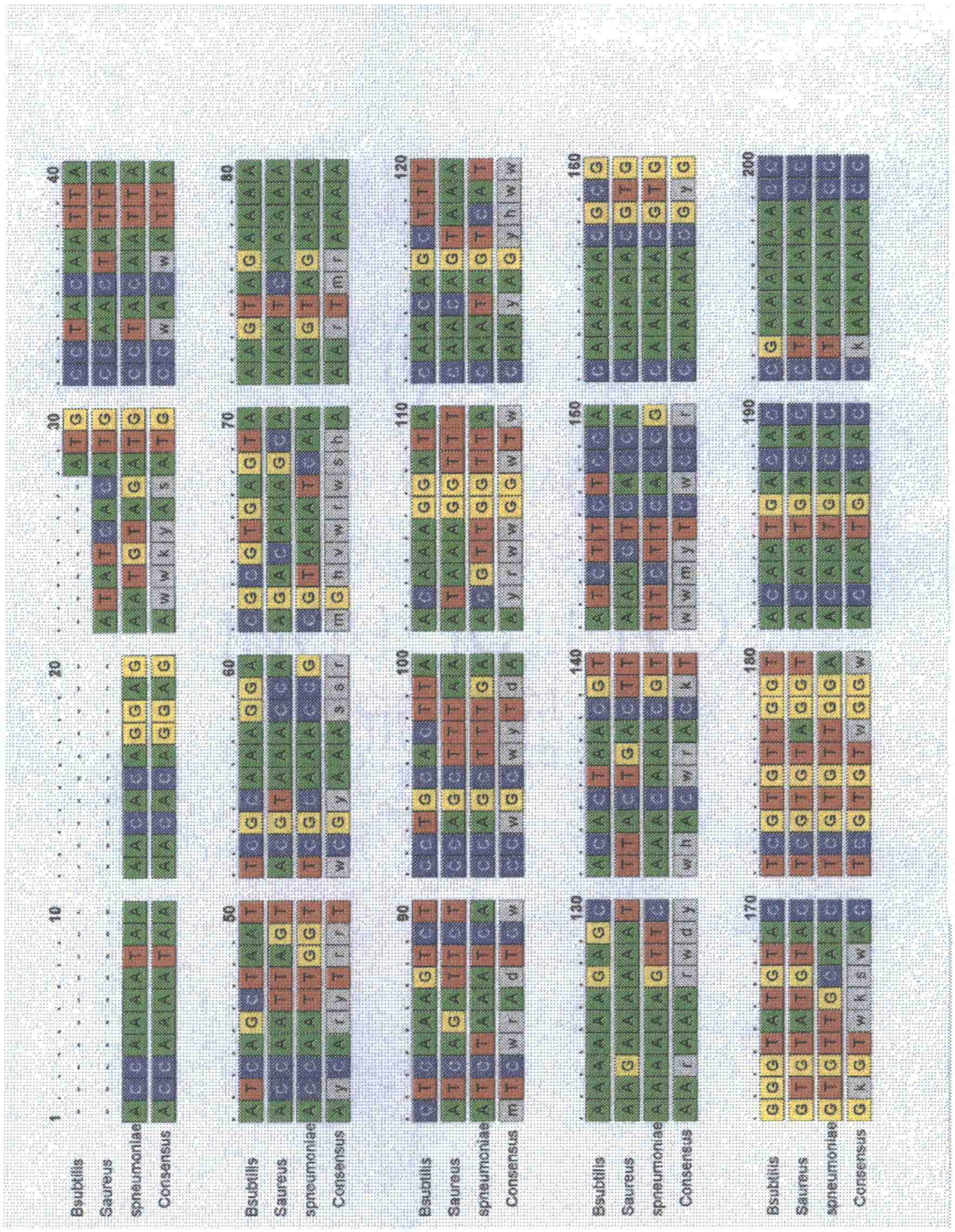


Figure 2.3.

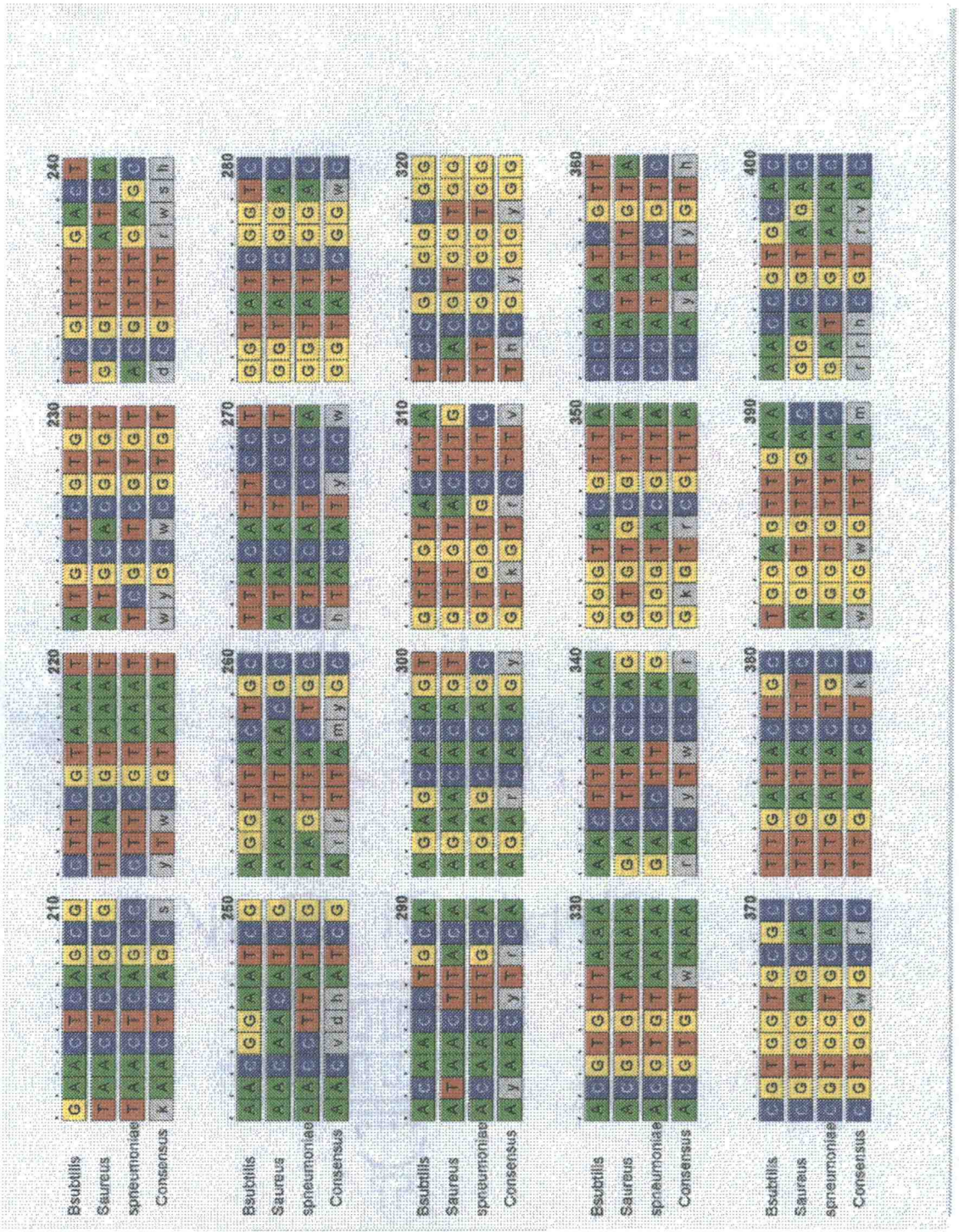


Figure 2.3. (Contd)

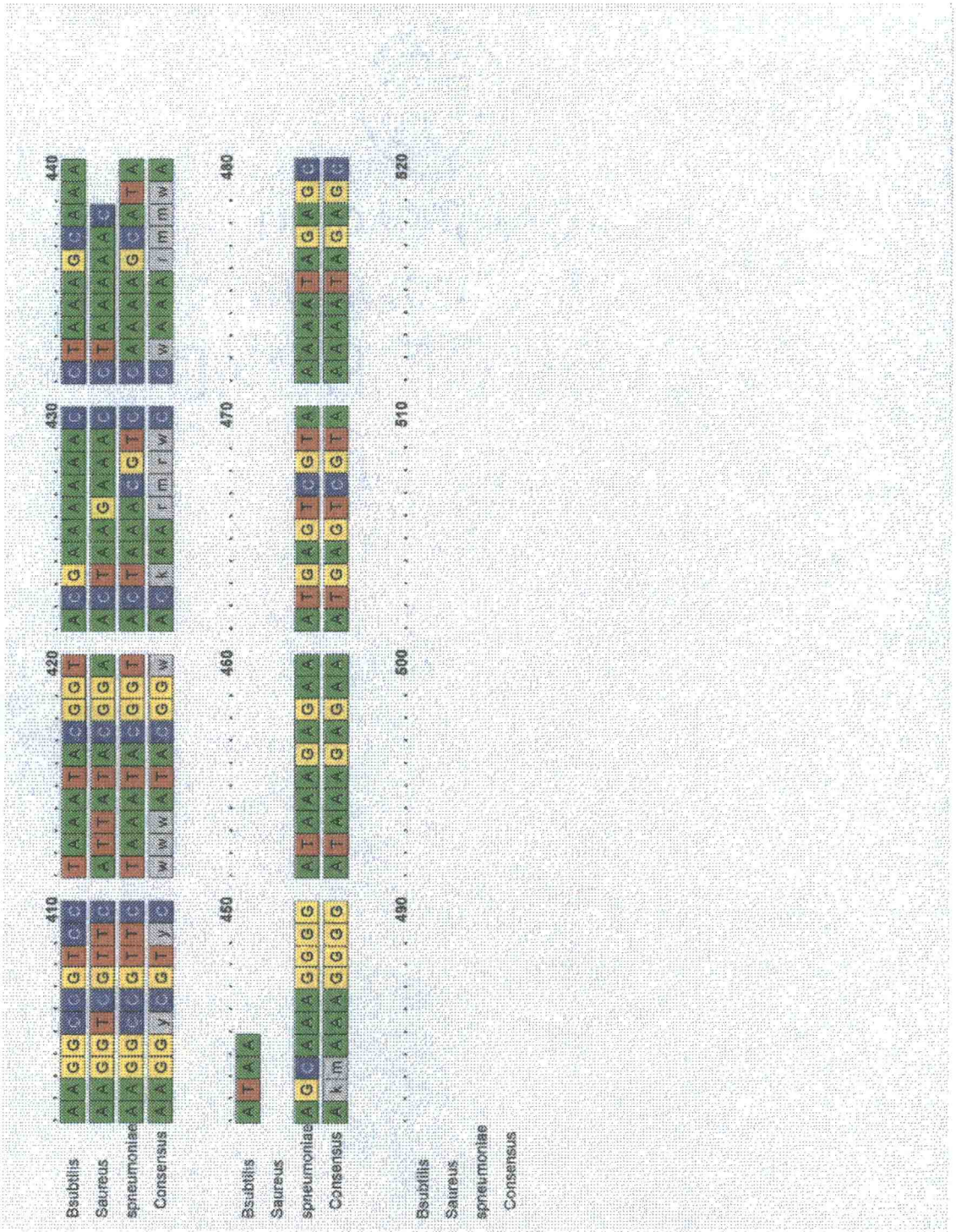


Figure 2.3. (Contd)

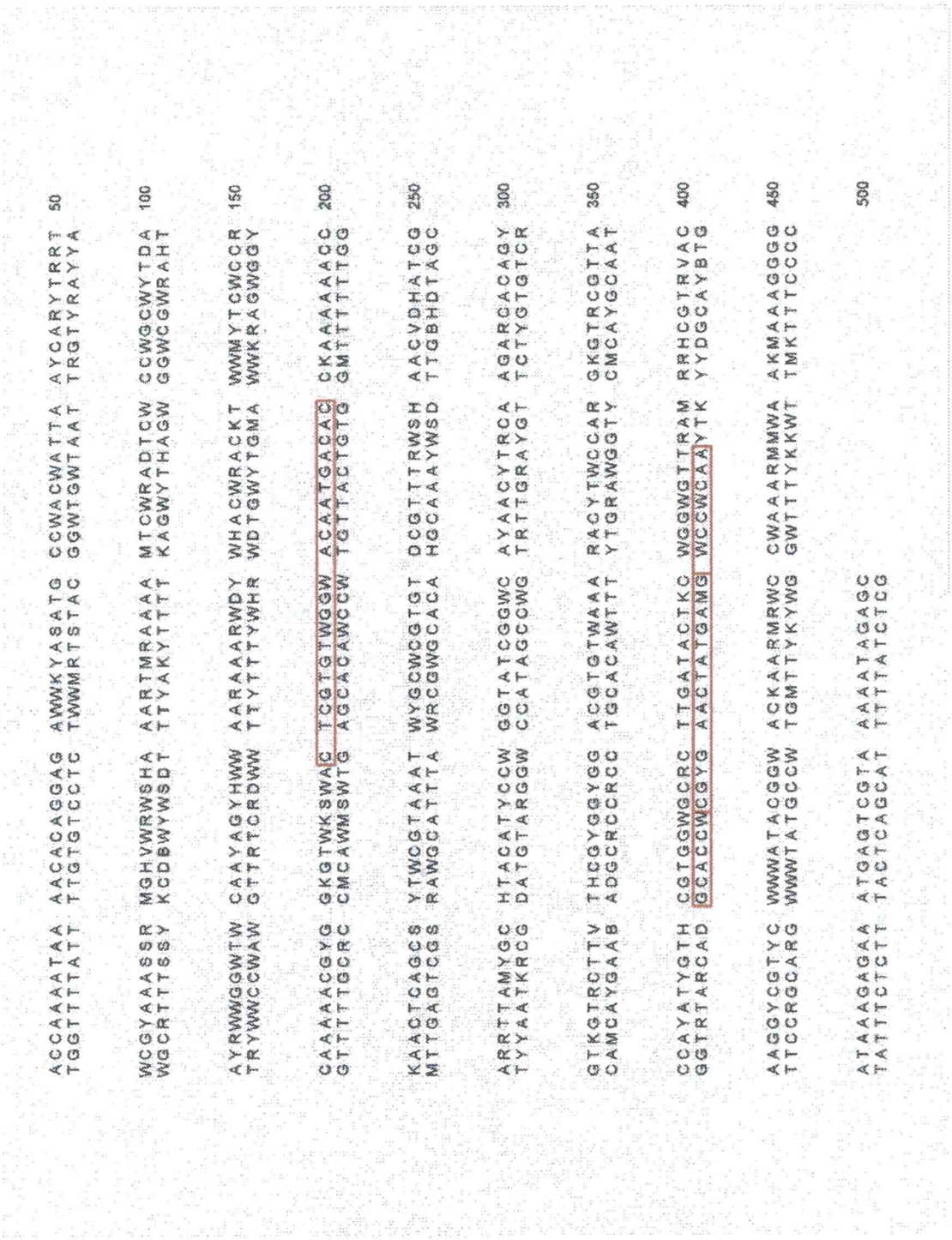


Figure 2.3(Contd)

The nucleotide sequences of the primers are listed in the Appendix.

Chromosomal DNA

Five milliliters of an bacterial overnight culture, 5 ml was pelleted and suspended in 50µl of water. This tube was placed in a boiling water bath for 10 minutes. The tube was centrifuged for 10 minutes at 15K rpm. The supernatant containing the chromosomal DNA was collected and quantitated using a fluorimeter and visualized on an agarose gel.

Except when stated, all steps were carried out using standard procedures (Sambrook et al., 1989). PCR reactions contained 20 pmol of each primer, CF56 and CF57 or CF58 and 120 ng of chromosomal DNA in the manufacturer's buffer. The PCR used 3 units of *Pfu* thermostable DNA polymerase (Promega, Madison, WI) in over 30 cycles of 94°C for 30 seconds, 50°C for one minute and 72°C for 2 minutes. A pre-incubation cycle, with 94°C for 1 minute, 47°C for 1 minute and 72°C for 2 minutes was

performed. The PCR product was cleaned using a QIAQuick kit and then sequenced.

Multiplex restriction site PCR (mRS PCR)

Three downstream primers (RV1D, RV2D, RV3D) and three upstream primers (RV1U, RV2U, RV3U) from the known sequence of the gene were designed. The relative orientation of these primers is shown in Figure 2.4. The primer on the unknown gene is a degenerate universal primer with a restriction enzyme site. More than one restriction enzyme primer can be used in one reaction, thus improving the probability of a successful gene walk (Sarkar et al., 1993). The outline of this method is given in Figure 2.5. The sequences of all the primers are listed in the Appendix.

200 pmol of the specific primer RV1D and 20 pmol of the restriction site primers were used in a 20 µl reaction. About 200 ng of the genomic DNA, 200 µM of

Figure 2.4.Formulation of primers for mRS PCR. This DNA was obtained as the PCR product of the primers CF56 and CF57 on the *S. gordonii* genomic DNA. The primers used in the mRS PCR gene walk are designed from this 210 bp DNA. RV1D, RV2D and RV3D denote primers designed to provide the downstream sequences in the gene walk and RV1U, RV2U, and RV3U are the primers to amplify the upstream sequences. The complete sequences of these primers are given in the Appendix.

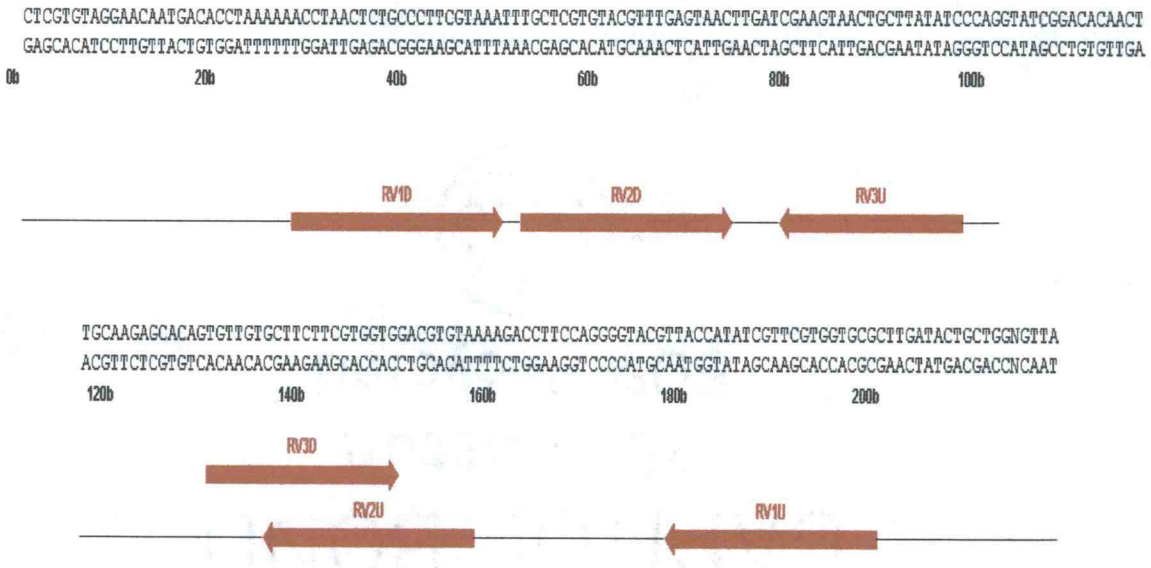
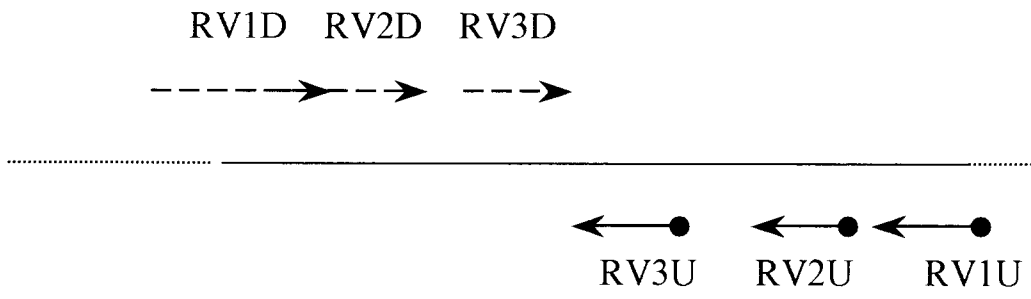

CTCGTGTAGGAACAATGACACCTAAAAAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTTTGAGTAACTTGATCGAAGTAACTGCTTATATCCCAGGTATCGGACACAACT
GAGCACATCCTTGTTACTGTGGATTTTTTGGATTGAGACGGGAAGCATTTAAACGAGCACATGCAAACTCATTGAACTAGCTTCATTGACGAATATAGGGTCCATAGCCTGTGTTGA
0b
20b
40b
60b
80b
100b
RV1D
RV2D
RV3U
TGCAAGAGCACAGTGTTGTGCTTCTTCGTGGTGGACGTGTAAAAGACCTTCCAGGGGTACGTTACCATATCGTTCGTGGTGCGCTTGATACTGCTGGNGTTA
ACGTTCTCGTGTCACAACACGAAGAAGCACCACCTGCACATTTTCTGGAAGGTCCCCATGCAATGGTATAGCAAGCACCACGCGAACTATGACGACCNCAAT
120b
140b
160b
180b
200b
RV3D
RV2U
RV1U

Figure 2.4.

Figure 2.5. The various steps in multiplex restriction site PCR. RV1D, RV2D AND RV3D denote primers designed to fetch the downstream sequences in the gene walk and RV1U, RV2U, and RV3U are the primers to amplify the upstream sequences. The sequences of the primers are given in the appendix.



PCR with RV1D and All Restriction primers

PCR with RV2D and All Restriction primers

Sequence with RV3D for the downstream sequence. Repeat with RV1U, RV2U and RV3U for upstream sequences.

Figure 2.5.

dNTP and 1.5 mM of $MgCl_2$ and 0.5 units of Amplitaq (Perkin Elmer, Branchburg, New Jersey) completed the reaction. Forty cycles comprised of 94°C for 1 min, 55° C for 2 min and 72°C for 3 min, were conducted. Then 1 µl of this end product was the template for PCRII. PCRII is similar to PCRI and RV2D was used in lieu of RV1D. The PCR product was cleaned and sequenced with RV3D. The same reactions were repeated with specific primers RVIU, RV2U and RV3U(PCR methods and applications(318-322:1993)).

Sequencing

All the sequencing was done at The Center for Gene Research and Biotechnology (CGRB) central services lab at Oregon State University. This facility uses the enzymatic method of DNA sequencing (Sanger et al, 1989). The sequences thus obtained were analyzed in the software program DNAid and Omiga. These software help in aligning the sequences, and also in translating the sequences and other such functions.

Cloning

The aim of this experiment was to clone the *rpsL* gene into the bacterium to observe the phenotypes (Figure 2.6). A plasmid was designed for this purpose. The plasmid $pTRKH_2$ was selected, as this plasmid was most suitable to transform into *S. gordonii* (O'Sullivan et al., 1992). The plasmid $pTRKH_2$ contains the erythromycin resistance gene as marker and gram-positive and gram-negative origins of replication. This plasmid does not contain a gram-positive promoter. The promoter 635 from *S. gordonii*, (Christine Franke and Tove' Bolken, Siga Pharmaceuticals, personal communication), which is 239 bp long, was isolated from the *S. gordonii* chromosomal DNA, using the primers RVprm1 and RVprm2. As large quantities of the S12 protein is mildly toxic to the cells (Timms et al., 1993), a hairpin loop was integrated at the beginning of the promoter such that the transcription and translation of the transformed gene was low. The promoter DNA and the plasmid were ligated such that the promoter was ligated in front of the multi-cloning site (MCS) of the plasmid in the 5' -> 3' direction, between the BglII and BamHI sites. The *rpsL* gene from the

various strains of *S. gordonii* was pulled out using the primers pTRKPstI and pTRKBamHI -(sequences in the Appendix) using PCR based methodologies. The plasmid pTRKH2 with the gram-positive promoter and the *rpsL* gene were each cut with restriction enzymes PstI and BamHI. The *rpsL* gene was ligated into the MCS. The rpsL gene from the strain V288 was transformed into the strain GP204 and the rpsL gene from GP204 was transformed into V288. The transformed bacteria were first selected on erythromycin (5 µg/ml) and then selected on streptomycin (500 µg/ml). Selection on two antibiotics in the same step caused undue stress on the bacterium and hence a single selection at a time was used.

Figure 2.6. Steps in cloning and transforming the rpsL gene from streptomycin resistant and streptomycin sensitive *S. gordonii* strains. All restriction enzymes wee from New England Biolabs and used with manufacturer's buffers.

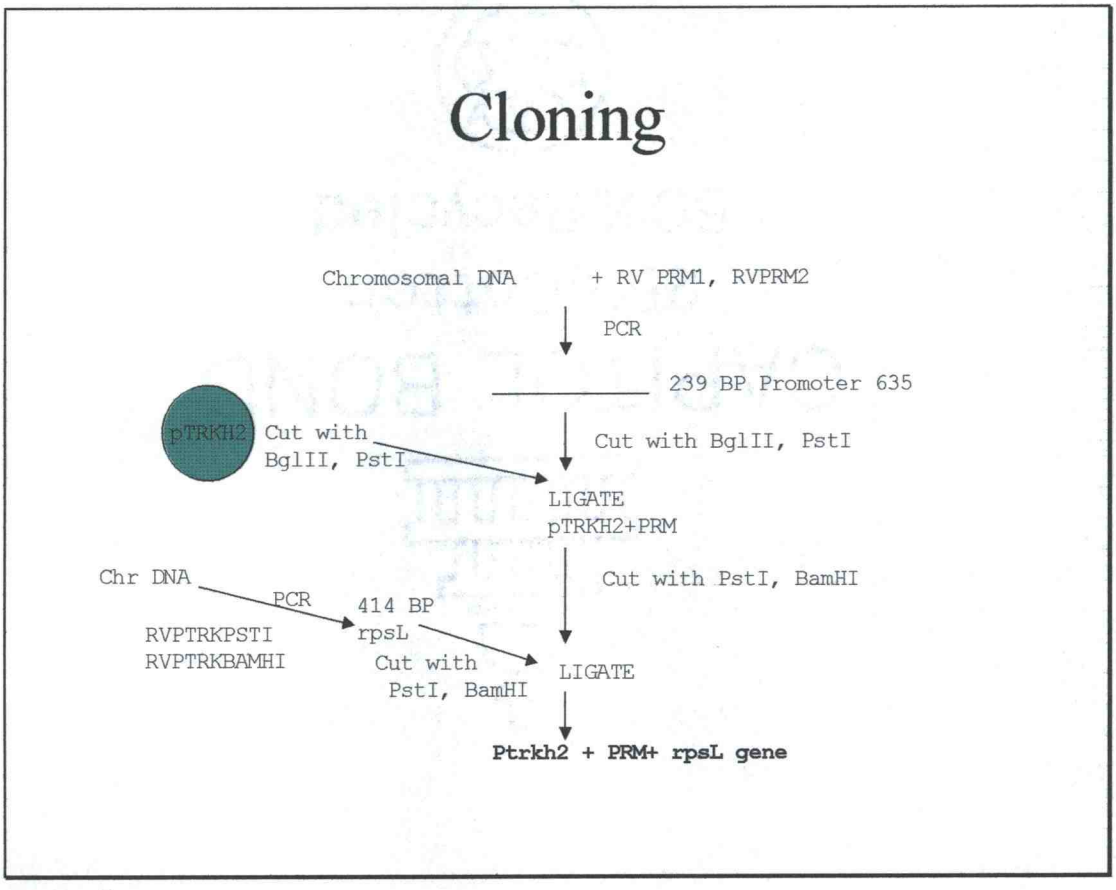

Cloning
Chromosomal DNA
+ RV PRM1, RVPRM2
PCR
239 BP Promoter 635
pTRKH2
Cut with
BglII, PstI
Cut with BglII, PstI
LIGATE
pTRKH2+PRM
Chr DNA
PCR
414 BP
rpsL
RVPTRKPSTI
RVPTRKBAMHI
Cut with
PstI, BamHI
Cut with PstI, BamHI
LIGATE
Ptrkh2 + PRM+ rpsL gene

Figure 2.6.

.

Results

The genomic DNA from strains V288, GP204, SP204 (1-1), and SP635 were subjected to PCR using the primers CF56 and CF57 or CF58. The gel analysis showed identical PCR products as seen in Figure 2.7. These PCR products upon sequencing, gave a 210 base pair DNA fragment, which was identical in all the four different strains of *S. gordonii*. The 210 bp fragment sequence is shown in Figure 2.8. This fragment aligned very well with the known S12 sequences from various bacteria and particularly, the S12 sequence from *S. pneumoniae* (Salles et al., 1993), a related gram-positive bacterium.

Using this sequence, the primers for mRS PCR were designed (Figure 2.4). The end product of mRS PCR is visualized on a 1.2% agarose gel in Figure 2.9. Some of the mRS PCR had multiple bands, probably because more than one restriction primer formed products. When this happened, each restriction primer was used separately and the products analyzed further. These PCR products were sequenced. Combining the sequences from the upstream and downstream primers and the 210 bp fragment from the

Figure 2.7. 1% Agarose Gel with the PCR products of *S. gordonii* strains with primers CF56 and CF57. This gel was stained with ethidium bromide. Lane 1 had the PCR product with V288 ``Challis'' strain as the DNA template. In lanes 2, 3 and 4 were the PCR products with the GP204, SP204(1-1) and SP635 templates respectively. Lane M was the 1 kb DNA ladder (MBI Fermentas).

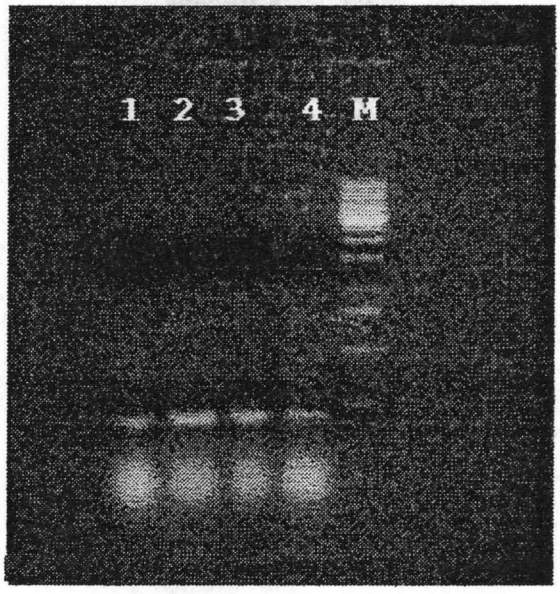


Figure 2.7.

Figure 2.8.The sequence from the PCR product using primers CF56 and CF57. These sequences were identical in the streptomycin sensitive V288 and the streptomycin resistant strain GP204, except for a single base, an A to C, which is highlighted. The first row is the sequence from the strain V288 and the second row is from the strain GP204.

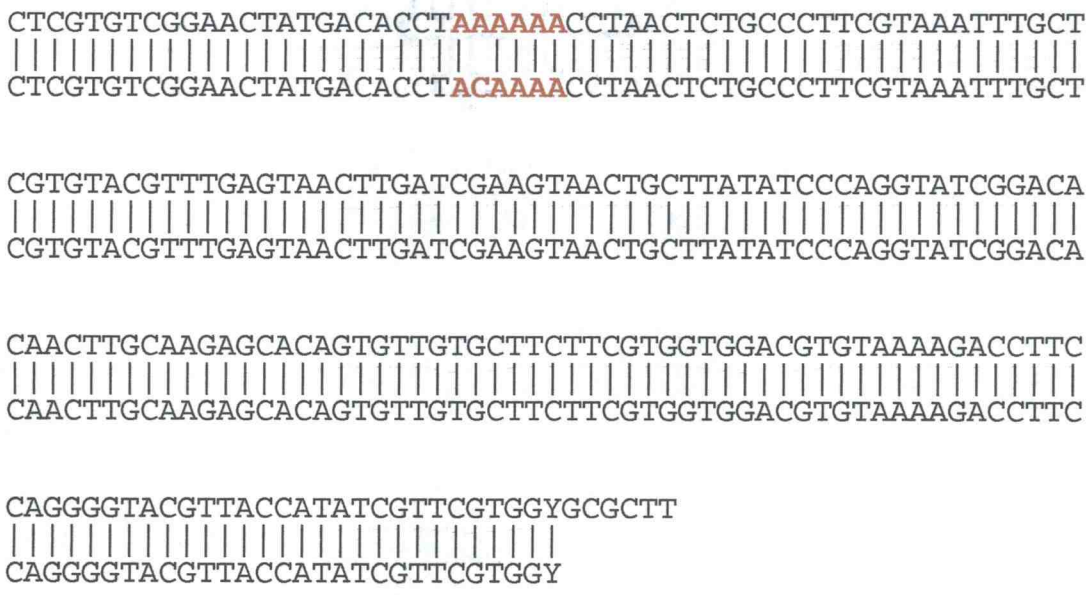

CTCGTGTCGGAACTATGACACCTAAAAAACCTAACTCTGCCCTTCGTAAATTTGCT
CTCGTGTCGGAACTATGACACCTACAAAACCTAACTCTGCCCTTCGTAAATTTGCT
CGTGTACGTTTGAGTAACTTGATCGAAGTAACTGCTTATATCCCAGGTATCGGACA
CGTGTACGTTTGAGTAACTTGATCGAAGTAACTGCTTATATCCCAGGTATCGGACA
CAACTTGCAAGAGCACAGTGTTGTGCTTCTTCGTGGTGGACGTGTAAAAGACCTTC
CAACTTGCAAGAGCACAGTGTTGTGCTTCTTCGTGGTGGACGTGTAAAAGACCTTC
CAGGGGTACGTTACCATATCGTTCGTGGYGCGCTT
CAGGGGTACGTTACCATATCGTTCGTGGY

Figure 2.8.

Figure 2.9. 1.2% agarose gel showing the end products of mRS (multi restriction site) PCR. The gel was stained with ethidium bromide. The template DNA used were the V288 and GP204 strains of *S. gordonii*. The primers used in mRS PCR I and II are RV1U and RV2U respectively. The lanes are marked on the gel. The last gel has a 1-10 Kb ladder (MBI Fermentas).

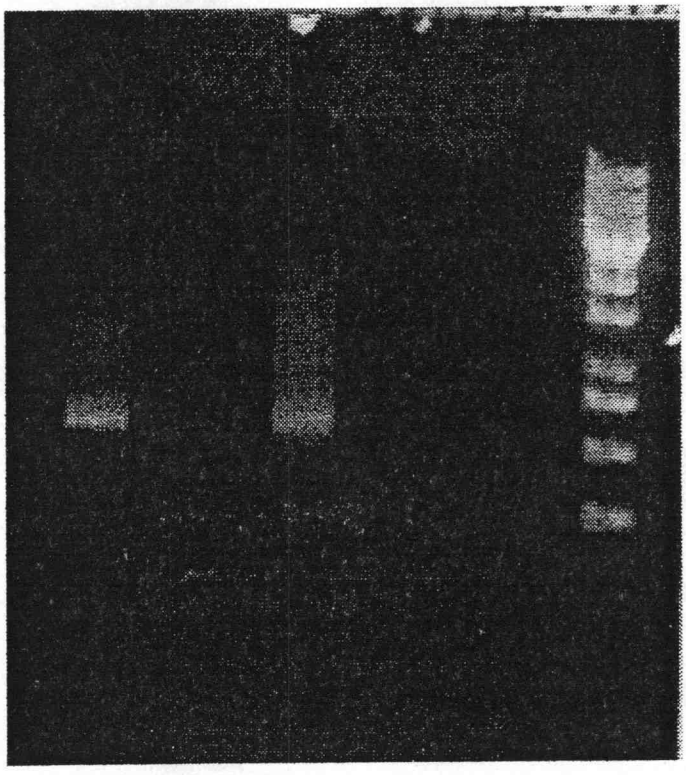


Figure 2.9.

earlier PCR using primers CF56 and CF57, the entire *rpsL* gene sequence was reconstructed via gene walking techniques. The gene sequences of the four strains are given in Figure 2.10.

The primers RVrpsl5′ and RVrpsl3′ were designed so that the full gene can be amplified using PCR and the sequence confirmed. The primers' sequences are given in the Appendix. These sequences were analyzed and the differences between the streptomycin resistant and streptomycin sensitive strains were thus elucidated.

In transformation experiments, the strain V288 transformed with the *rpsL* gene of GP204 remained streptomycin sensitive. The streptomycin resistant GP204 strain transformed with the *rpsL* gene of strain V288 became streptomycin sensitive. The number of colonies from this transformation was less and colonies, which were resistant to lower concentrations of streptomycin, were also found.

Figure 2.10. The sequence of S12 gene from the strains V288, GP204, SP204 (1-1) and SP635 of *S. gordonii*. The sequence, which contains the coding sequence alone, has 414 base pairs. The sequences are identical except for the single base change, a point mutation in the base 167. The sequence in blue script is the sequence of the S12 gene from the *S. gordonii* strain V288. The sequence in green represents the S12 gene from the strain GP204, black from SP204 (1-1) and red from SP635. The region of interest is in bold letters.

Accession Number:

ATGCCTACAATTAACCAATTGGTTCGCAAACCGCGTAAATCAAAAGTAGAAAAATC
ATGCCTACAATTAACCAATTGGTTCGCAAACCGCGTAAATCAAAAGTAGAAAAATC
ATGCCTACAATTAACCAATTGGTTCGCAAACCGCGTAAATCAAAAGTAGAAAAATC
ATGCCTACAATTAACCAATTGGTTCGCAAACCGCGTAAATCAAAAGTAGAAAAATC

TAAATCACCAGCATTGAACGTTGGTTACAATAGTTTGAAACGTGTTCCAACTAACG
TAAATCACCAGCATTGAACGTTGGTTACAATAGTTTGAAACGTGTTCCAACTAACG
TAAATCACCAGCATTGAACGTTGGTTACAATAGTTTGAAACGTGTTCCAACTAACG
TAAATCACCAGCATTGAACGTTGGTTACAATAGTTTGAAACGTGTTCCAACTAACG

AAAGTGCACCTCAAAAACGTGGTGTAGCGACTCGTGTCGGAACTATGACACCTAAA
AAAGTGCACCTCAAAAACGTGGTGTAGCGACTCGTGTCGGAACTATGACACCTACA
AAAGTGCACCTCAAAAACGTGGTGTAGCGACTCGTGTCGGAACTATGACACCTACA
AAAGTGCACCTCAAAAACGTGGTGTAGCGACTCGTGTCGGAACTATGACACCTACA

AAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTTTGAGTAACTTGATCGA
AAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTTTGAGTAACTTGATCGA
AAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTTTGAGTAACTTGATCGA
AAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTTTGAGTAACTTGATCGA

AGTAACTGCTTATATCCCAGGTATCGGACACAACTTGCAAGAGCACAGTGTTGTGC
AGTAACTGCTTATATCCCAGGTATCGGACACAACTTGCAAGAGCACAGTGTTGTGC
AGTAACTGCTTATATCCCAGGTATCGGACACAACTTGCAAGAGCACAGTGTTGTGC
AGTAACTGCTTATATCCCAGGTATCGGACACAACTTGCAAGAGCACAGTGTTGTGC

TTCTTCGTGGTGGACGTGTAAAAGACCTTCCAGGGGTACGTTACCATATCGTTCGT
TTCTTCGTGGTGGACGTGTAAAAGACCTTCCAGGGGTACGTTACCATATCGTTCGT
TTCTTCGTGGTGGACGTGTAAAAGACCTTCCAGGGGTACGTTACCATATCGTTCGT
TTCTTCGTGGTGGACGTGTAAAAGACCTTCCAGGGGTACGTTACCATATCGTTCGT

GGTGCGCTTGATACAGCAGGTGTTACTGATCGTAAACAAGGCCGTTCTAAATACGG
GGTGCGCTTGATACAGCAGGTGTTACTGATCGTAAACAAGGCCGTTCTAAATACGG
GGTGCGCTTGATACAGCAGGTGTTACTGATCGTAAACAAGGCCGTTCTAAATACGG
GGTGCGCTTGATACAGCAGGTGTTACTGATCGTAAACAAGGCCGTTCTAAATACGG

TACTAAAAAACCAAAAGCATAA
TACTAAAAAACCAAAAGCATAA
TACTAAAAAACCAAAAGCATAA
TACTAAAAAACCAAAAGCATAA

Figure 2.10.

Discussion

Analyzing the gene sequences of GP204, SP204 and SP635, all three sequences were found to be identical. The GP204 sequence was used for further analysis. The putative rpsL gene open reading frame of *S. gordonii* contains 414 coding nucleotides including the stop codon and 136 amino acid codons. Comparing the sequences of *S. gordonii* strains V288 and GP 204, they were different only in the identity of the residue at the position 167. The sequence AAAAAA in V288 compares as ACAAAA in GP204. In the S12 protein, the mutation AAA to ACA translated to a change from lysine to threonine in the amino acid 56. The nucleotide comparison is depicted in Figure 2.11A and the comparison of the protein sequence in Figure 2.11B.

It was shown in *E.coli* that Sm^d mutants involved a change from proline to leucine at position 90 or glycine to aspartic acid at 91. In Sm^r mutants the changes were either a change from lysine to arginine at position 87 or lysine to arginine, asparagine or threonine at 42 (Funatsu and Wittman, 1972, Yaguchi et al., 1975 and Funatsu et al., 1977).

Figure 2.11A. Comparison of the nucleotide sequences of *S.gordonii* strains V288 and GP204. The first row to be compared is GP204 and the lower row is V288. The area of interest is highlighted and the amino acid composition of this region is given along the nucleotide sequence.
Figure 2.11B. The S12 protein sequences of *S. gordonii* strains V288 and GP204 are analyzed by comparison. The first row of amino acids represents the S. gordonii strain V288 and the bottom row represents the strain GP 204. The lysine 56 of V288 is mutated to threonine in GP204, the putative cause of streptomycin resistance.

```
ATGCCTACAATTAACCAATTGGTTCGCAAACCGCGTAAATCAAAAGTAGAAA
|||||||||||||||||||||||||||||||||||||||||||||||||||||
ATGCCTACAATTAACCAATTGGTTCGCAAACCGCGTAAATCAAAAGTAGAAA

AATCTAAATCACCAGCATTGAACGTTGGTTACAATAGTTTGAAACGTGTTCC
|||||||||||||||||||||||||||||||||||||||||||||||||||||
AATCTAAATCACCAGCATTGAACGTTGGTTACAATAGTTTGAAACGTGTTCC

AACTAACGAAAGTGCACCTCAAAAACGTGGTGTAGCGACTCGTGTCGGAAC
|||||||||||||||||||||||||||||||||||||||||||||||||||||
AACTAACGAAAGTGCACCTCAAAAACGTGGTGTAGCGACTCGTGTCGGAAC

         P  T  K  P
TATGACACCTACAAAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTT
|||||||||||| |||||||||||||||||||||||||||||||||||||||
TATGACACCTAAAAAACCTAACTCTGCCCTTCGTAAATTTGCTCGTGTACGTT
         P  K  K  P

TGAGTAACTTGATCGAAGTAACTGCTTATATCCCAGGTATCGGACACAACTT
|||||||||||||||||||||||||||||||||||||||||||||||||||||
TGAGTAACTTGATCGAAGTAACTGCTTATATCCCAGGTATCGGACACAACTT

GCAAGAGCACAGTGTTGTGCTTCTTCGTGGTGGACGTGTAAAAGACCTTCC
|||||||||||||||||||||||||||||||||||||||||||||||||||||
GCAAGAGCACAGTGTTGTGCTTCTTCGTGGTGGACGTGTAAAAGACCTTCC

AGGGGTACGTTACCATATCGTTCGTGGTGCGCTTGATACAGCAGGTGTTAC
|||||||||||||||||||||||||||||||||||||||||||||||||||||
AGGGGTACGTTACCATATCGTTCGTGGTGCGCTTGATACAGCAGGTGTTAC

                                                      414
TGATCGTAAACAAGGCCGTTCTAAATACGGTACTAAAAAACCAAAAGCATAA
|||||||||||||||||||||||||||||||||||||||||||||||||||||
TGATCGTAAACAAGGCCGTTCTAAATACGGTACTAAAAAACCAAAAGCATAA
                                                      414
```

Figure 2.11A.

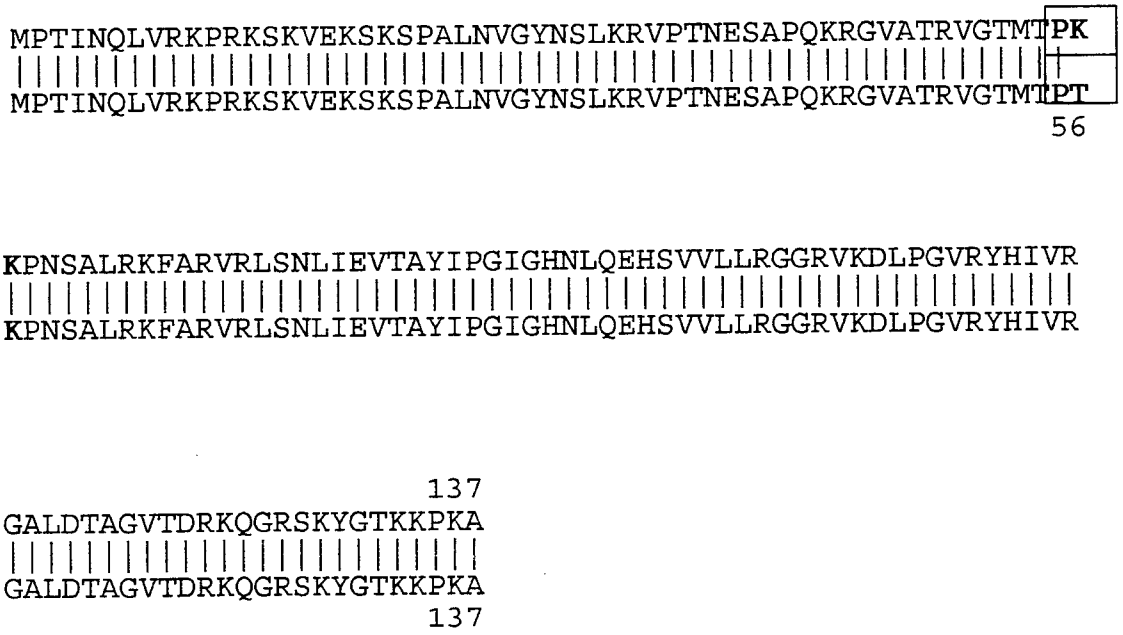

MPTINQLVRKPRKSKVEKSKSPALNVGYNSLKRVPTNESAPQKRGVATRVGTMTPK
MPTINQLVRKPRKSKVEKSKSPALNVGYNSLKRVPTNESAPQKRGVATRVGTMTPT
56
KPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLLRGGRVKDLPGVRYHIVR
KPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLLRGGRVKDLPGVRYHIVR
137
GALDTAGVTDRKQGRSKYGTKKPKA
GALDTAGVTDRKQGRSKYGTKKPKA
137

Figure 2.11B.

In *Mycobacterium tuberculosis*, streptomycin resistance was caused by the lysine codon in position 43 to arginine (Honore and Cole, 1994). In another study, 41 streptomycin resistant *M. tuberculosis* strains were studied and 49% were attributed to a mutation in the *rpsL* gene. In these, 70% were changes in lysine cordon 43 (Finken et al., 1993; Meier et al., 1994). In *M. smegmatis*, streptomycin resistance was attributed to lysine 43 and lysine 88 (analogous to 42 and 87 respectively in *E.coli*) (Kenney et al., 1994). *Streptococcus pneumoniae str41* allele, which has high streptomycin resistance, has been well studied as a reference marker. The *str 41* mutations corresponded to a single base substitution, converting lysine (AAA) to threonine (ACA) at position 56 (Salles et al., 1992), similar to our observed results.

Comparing the sequences of *E. coli* and *S. gordonii*, conserved regions are seen, as shown in Figure 2.12. The predicted S12 protein of S. gordonii is 68% identical to *E. coli* S12 protein and 95.7% identical to *S. pneumoniae* S12 protein. This is seen in Figure 2.13, where the S12 protein of *S. gordonii* and *S. pneumoniae* are compared.

Figure 2.12. Analyzing the protein sequence of the S12 protein of *S. gordonii* (V288) and the S12 protein of *E.coli*.
The *E. coli* sequence is from the genbank accession no V00355. Ec denotes the E.coli S12 protein sequence and Sg symbolizes the *S. gordonii* V288 S12 sequence. The dotted lines in the *E.coli* (--) denote gap in the sequence to optimize the alignment. The lysine 56 to threonine change corresponding to the streptomycin resistance is indicated by the bold typeface.

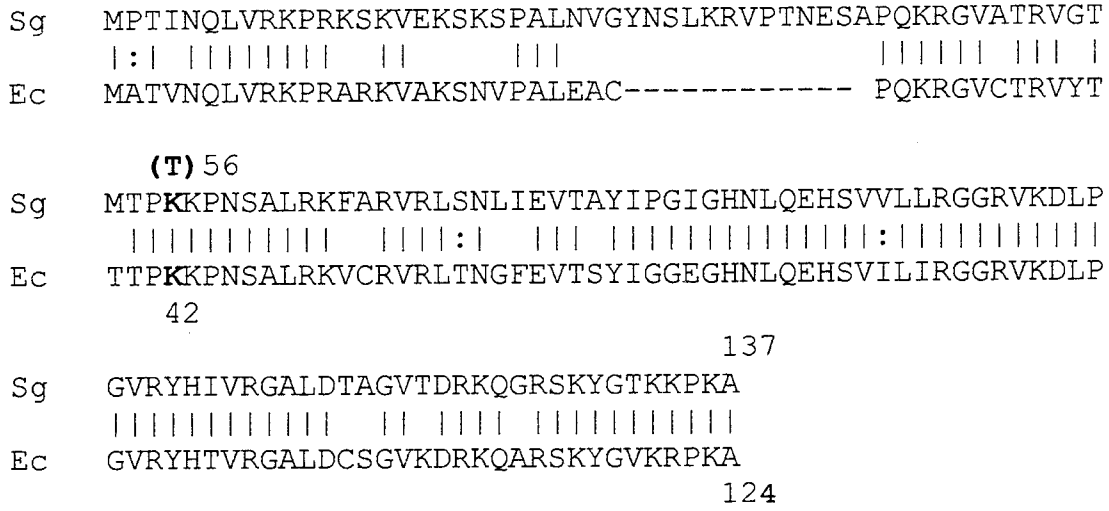

Sg MPTINQLVRKPRKSKVEKSKSPALNVGYNSLKRVPTNESAPQKRGVATRVGT
Ec MATVNQLVRKPRARKVAKSNVPALEAC---------- PQKRGVCTRVYT
(T)56
Sg MTPKKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLLRGGRVKDLP
Ec TTPKKPNSALRKVCRVRLTNGFEVTSYIGGEGHNLQEHSVILIRGGRVKDLP
42
137
Sg GVRYHIVRGALDTAGVTDRKQGRSKYGTKKPKA
Ec GVRYHTVRGALDCSGVKDRKQARSKYGVKRPKA
124

Figure 2.12.

Figure 2.13. Comparison of the S12 protein sequence of *S. gordonii* (V288) and the sequence of S12 protein of *S.pneumoniae*. The first row, labeled Sp, represents the S12protein sequence from *S. pneumoniae*. The row marked Sg is the S12 protein sequence of *S. gordonii* V288 strain. There is over 95% homology between the S12 sequences.

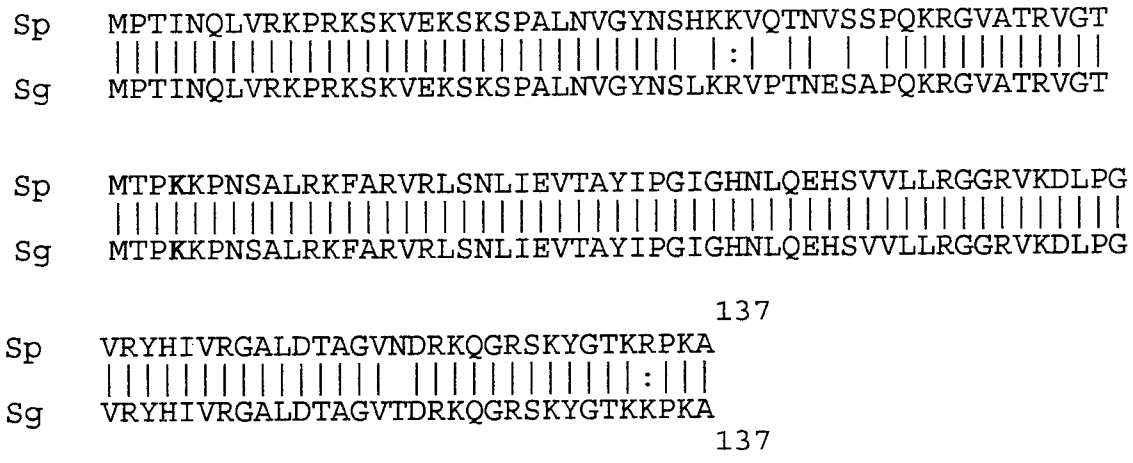

Sp MPTINQLVRKPRKSKVEKSKSPALNVGYNSHKKVQTNVSSPQKRGVATRVGT
Sg MPTINQLVRKPRKSKVEKSKSPALNVGYNSLKRVPTNESAPQKRGVATRVGT
Sp MTPKKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLLRGGRVKDLPG
Sg MTPKKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLLRGGRVKDLPG
137
Sp VRYHIVRGALDTAGVNDRKQGRSKYGTKRPKA
Sg VRYHIVRGALDTAGVTDRKQGRSKYGTKKPKA
137

Figure 2.13.

The amino acid 56 of *S. gordonii* corresponded to the amino acid 42 in *E. coli*. The amino acid 42 (lysine) in the *E.coli* S12 sequence which when replaced by one of the three amino acids arginine, asparagine or threonine results in high level streptomycin resistance (Tubulekas et al., 1991). It can be concluded that the mutation in lysine 56 is similar to the mutation in lysine 42 of *E. coli*.

The lysine to threonine mutation in *S. gordonii* apparently causes streptomycin resistance as three independent isolates all contained the same mutation. This follows what has been seen in many other bacteria. As the S12 protein is a conserved protein and essential for bacterial viability, it is important that no mutation disrupts its function. As seen in the literature review, some of these mutations in the lysine cause hyper accurate translation or slow rate of elongation and leading to slower growth (Kashiwagi et al., 1998) (Saito et al., 1994) (Timms et al., 1993). In *E. coli*, the change of lysine to arginine was non-restrictive. The mutation of lysine to threonine showed modest increase in doubling time, from 42 minutes to 54.7 minutes (Tubelekas et al., 1993). The elongation rate (amino acids/sec) was

12.6 for the wild type and 8.9 for the mutant, which had lysine 42 to threonine (Tubulekas et al., 1993). Looking at this data and from our observations, we can speculate that this mutation in *S. gordonii* does not cause any debilitating effect on the bacterium, in the absence of drug selection.

The streptomycin sensitive wild type strain V288 remained streptomycin sensitive when transformed with the *rpsL* gene from the streptomycin resistant strain GP204. This confirmed the observations seen in literature that the streptomycin sensitive wild type is dominant. Similarly, the streptomycin resistant GP204 strain, when transformed with the wild type *rpsL* gene, became streptomycin sensitive. Due to the low number of colonies isolated and due to the presence of colonies that are resistant to streptomycin in lower concentrations, this experiment needs to be repeated in the future, to clarify these results.

In summary, there is a mutation in amino acid 56 of the S12 protein of *S. gordonii* changing lysine to threonine. This mutation apparently causes streptomycin resistance in the bacterium. This mutation in S12

protein follows a pattern seen in the S12 proteins from other streptomycin-resistant bacteria.

CHAPTER 3
STREPTOMYCIN RESISTANT MUTANTS IN *STREPTOCOCCUS PYOGENES*

Introduction

Streptococcus pyogenes is a gram-positive pathogenic bacteria. It causes a wide spectrum of diseases, from pharyngitis (or sore throat) to rheumatic fever, glomerulo nephritis and necrotizing fasciitis. It is difficult to determine what causes one individual to have a simple sore throat and for another individual to develop rheumatic heart disease. Although particular strains are correlated with a certain disease type, it is still not totally predictable. The bacterium has a vast repertoire of antigens and more than 200 serotypes exist. The most important of these antigens is the M protein. M protein has been discussed in the Introduction. Two important factors in *S. pyogenes* pathogenicity are the M protein and the bacterial adhesion to the pharyngeal cell or the ease with which the bacterium attaches to the throat. Adhesion plays a key role in the bacterial pathogenicity. In order to be a successful pathogen, *S.*

pyogenes has to attach to the throat epithelial cells and multiply before the immune system of the host attacks it. The adhesion is particularly important because without adhesion, the bacterium will be readily eliminated by the innate immune system of the host. The amount of M protein present defines pathogenicity. The presence or absence of the M protein and the amount of M protein on the cell surface also determine the antigenic serotype of the organism. Thus these two factors give an indication of pathogenicity in *S. pyogenes*. In this study, these two factors are tested as signs of virulence in streptomycin resistant *S. pyogenes* strains.

Materials and Methods

Strains Used

The *S. pyogenes* strains used in this study are the S43ATCC, S43/192/4, S43/192/4P8, and S43/192/30R strains. The S43ATCC strain was the S43 strain from the ATCC stock. The S43/192/4 is a *S. pyogenes* strain from the Rockefeller collection. This strain was made

streptomycin resistant and referred to as S43/192/4SmR. S43/192/4P8 is the S43/192/4SmR strain, which was passaged 8 times in mice. S43/192/30R referred to in this study is another strain of *S. pyogenes* from the Rockefeller collection. This strain is streptomycin resistant. These strains are listed in Table 3.1. Dr. Kevin Jones of SIGA Pharmaceuticals kindly provided these strains.

Colony Morphology

The strains were streaked on BHI agar (DIFCO) with suitable antibiotics. BHI is brain heart infusion to which is added 10% agar. Blood agar (BA) plates with antibiotics were also used. These blood agar plates were useful in monitoring hemolysis. With S43ATCC strain, no antibiotics were used. For all the streptomycin resistant strains, 500µg/ml of streptomycin

Table 3.1. Shows the strains used in this study. SmR denotes streptomycin resistance and when no symbol is present, it denotes the wild type (no streptomycin resistance). Other additional features are mentioned.

strains	Streptomycin Resistance	Source	Additional Features
S43 ATCC	-	ATCC	
S43 ATCC SmR	+	Lab Strain	Strain was made streptomycin resistant in the lab following standard procedures [a].
S43/192/4	-	Dr. K Jones Rockefeller Collection [b].	
S43/192/4 SmR	+	Lab Strain	Strain was made streptomycin resistant in the lab following standard procedures [a]
S43/192/4 P8	+	Lab Strain	S43/192/4 SmR was passaged in mice 8 times [c].

Table 3.1

S43/192/30R	+	Dr. K Jones Rockefeller Collection [b].	

[a]-Reference Sambrook et al.,1989. [c]- Dr.K. Jones (Siga Pharmaceuticals)
[b]-Siga Pharmaceuticals

Table 3.1.(Contd)

was added. The plates were incubated at 37°C for 48 hours.

Polymerase Chain Reaction (PCR)

The PCR was done as described in Chapter 2. The primers CF56 and CF57 were used in the first study and part of the S12 gene was sequenced. Using the primers TT1 and TT2 the full gene was recovered and sequenced. The sequences of the primers are given in the appendix.

ImmunoBlot

The test strains were streaked on BHI plates with appropriate antibiotics and incubated overnight. A nitrocellulose membrane was applied on the plate for 20 minutes at room temperature. Then the membrane was incubated for 30 minutes at 37°C and for 15 minutes at 80°C. The membrane was blocked for 30 minutes with TBS (Tris buffered saline) to which 3% gelatin was added. The membrane was decanted and covered with TTBS (Tween20

wash solution in TBS)and shaken in a orbital shaker for 30 minutes. 20 ml of TTBS with 1% gelatin was added to 40 µl of 10F5, an anti M6 monoclonal antibody, and incubated at room temperature for 3 hours. The membrane was washed 3X with TTBS, and 20 ml of TTBS with 6.6 µl of secondary antibody conjugated with alkaline phosphate was added. This was incubated for 2 hours. The blot was washed twice with TTBS and once with TBS. The blot was then developed using the color developer solution.

ELISA (enzyme linked immunosorbent assay)

Competition ELISA was used to quantitate the amount of the M protein on the surface of the bacterium. The bacterial cells were prepared as described below and were reacted with a known amount of anti-M antibody. The anti M antibodies used were the monoclonal antibodies 10F5 and 10B6 (Jones et al., 1985). SIGA Pharmaceuticals provided these antibodies. The antibody, which was not bound to the antigen on the bacterial surface, was titrated against known concentrations of purified M protein.

The bacterial cultures were grown to A_{650nm} of 0.6 - 0.7. The cultures were then centrifuged, washed and resuspended in PBS (+0.02% sodium azide). To kill the cells, the cultures were placed at 56°C for 60 minutes. After washing and centrifuging, the O.D was adjusted to 1.0. These normalized cells were used in the competition ELISA.

In a V-bottom microtitre wells, 75 µl of PBS/Brij was dispensed in columns 2 -12. 150 µl of the bacterial suspension was added to the first well of each row in triplicate. Doubling dilutions down each row were performed by sequentially transferring 75 µl from the first wells to each succeeding well. 75 µl of the antibody was added and rotated at 4°C overnight. A flat bottom plate was coated with 100 µl diluted antigen in carbonate buffer (containing 0.02% sodium azide) and incubated at room temperature overnight. The antigen was removed from the flat bottom plate and washed 5X with PBS/Brij. The plate was blocked using 2% BSA in PBS/Brij for 1 hour at 37°C. The V- bottom plate was centrifuged for 15 min at 2000 rpm. The flat bottom plate was emptied and 50 µl from each well in the V bottom plate was

transferred carefully to the flat bottom plate. The flat bottom plate was incubated for 3 hours at 37°C. Then the plate was emptied and washed 6X with PBS/Brij. 50 µl of goat anti mouse secondary antibody (alkaline phosphatase conjugate) at 1:1000 dilution in PBS/Brij was added and incubated at 37°C for 2 hours. The plate was emptied and washed 5X. The plate was developed with 200 µl phosphatase substrate (Sigma) at 1 mg/ml in 10% diethanolamine/3 mM $MgCl_2$ (pH 10.0). The plate was read at 405 nm (Pozzi et al., 1992). The carbonate buffer, PBS, and other buffers used were prepared following standard procedures (Sambrook et al., 1989).

Pharyngeal cell adhesion

The overnight cultures of the *S. pyogenes* strains were washed twice with PBS containing 1mM $CaCl_2$ and 2mM $MgCl_2$). The bacterial cells were normalized at A_{650} to 1.

The control strain is the *S. pyogenes* strain (henceforth referred as the "luci" strain") containing the plasmid pCSS945. This plasmid contains the *lucGR*

gene from *Pyrophorus. plagiothalamus* and is chloramphenicol resistant (Loimaranta et al., 1998). Thus, the luci strain contains and expressed the luciferase gene in it.

The pharyngeal cells (pharyngeal carcinoma cell line, Detroit 562 ATCC CLL 138), were plated on a 16 well plate and washed with HEPES minimal essential media supplemented with 10% fetal calf serum. Various dilutions between the test strains, the luci strain and the medium were made. The dilutions were put on cells for 3 hours. The cells were washed with PBS(+ $CaCl_2$ and $MgCl_2$). To each of the wells was added 250 µl of D-luciferin (1mM D-luciferin in 100 mM sodium citrate buffer, pH 5.0). The plate was read immediately and the competitive adherence of the test strain against the luci strain and the resultant luminescence was measured.

Electron Microscopy

The overnight cultures of the bacterial strains were centrifuged at 3000Xg for 10 minutes. The pelleted cells

were washed thrice with PBS and then were suspended in PBS at 10^{10} cells/ml. Varying concentrations of the monoclonal antibody 10F5 were added. The ratio of 100 µl of the cell and 100 µl/ml of the antibody worked best. After incubation at 37°C for 60 minutes, the cells were centrifuged for 10 minutes at 1000Xg. The cells were washed with PBS to which is added 0.5% BSA. The final cell pellet was resuspended in 50 µl of PBS. The secondary antibody reaction with gold attached anti mouse IgG was set up in the ratio of 50:1 for 60 minutes at 37°C. The cells were washed, centrifuged and resuspended in 100 µl of PBS. These cells were added on a copper grid and the excess liquid blotted away and viewed under the electron microscope (Demuth et al., 1989).

Results

The colonies of the different strains of *S. pyogenes*, S43 ATCC, S43/192/4 SmR, and S43/192/30R were observed on the plates. All the three strains are streptomycin resistant and streaked on BHI plates with

500μg/ml. As seen in Figure 3.1, the colonies of S43/192/4 and S43/192/30R are strikingly different. S43/192/4 forms small, regular, convex white colonies. The 30R strain and the S43 strains form large mucoid colonies, which on further incubation becomes flat and dry. On blood agar plates, all the strains of *S. pyogenes* showed β-hemolysis.

The genomic DNA of the various strains of *S. pyogenes* was subjected to PCR using the primers TT1 and TT2. These primers were designed from the putative sequence of the *rpsL* gene of *S. pyogenes* from the genbank database (Chris Franke, SIGA Pharmaceuticals). After the S12 gene was amplified using PCR and cleaned using the QIAquick kit, these PCR products were sequenced. The sequences of streptomycin sensitive S43ATCC strain and streptomycin sensitive S43/192/4 were identical. The streptomycin resistant strain of S43ATCC, which was made streptomycin resistant in the lab using standard procedures, was also sequenced and the comparison between the streptomycin sensitive and resistant S43ATCC is given in Figure 3.3.

Figure 3.1. The S. pyogenes strains S43ATCC SmR, S43/192/4 SmR and S43/192/30R streaked on a BHI plate with the antibiotic streptomycin (500 µg/ml). The plate was incubated at 37°C for about 36 hours. The difference in the colony morphology is seen with the strains S43ATCC and S43/192/30R forming mucoid colonies whereas the S43/192/4 SmR strain forms convex, small, white and regular colonies.
In the plate, 1 denotes S43/192/4 SmR, 2 as the S43 ATCC strain and 3 denotes the strain S43/192/30R.

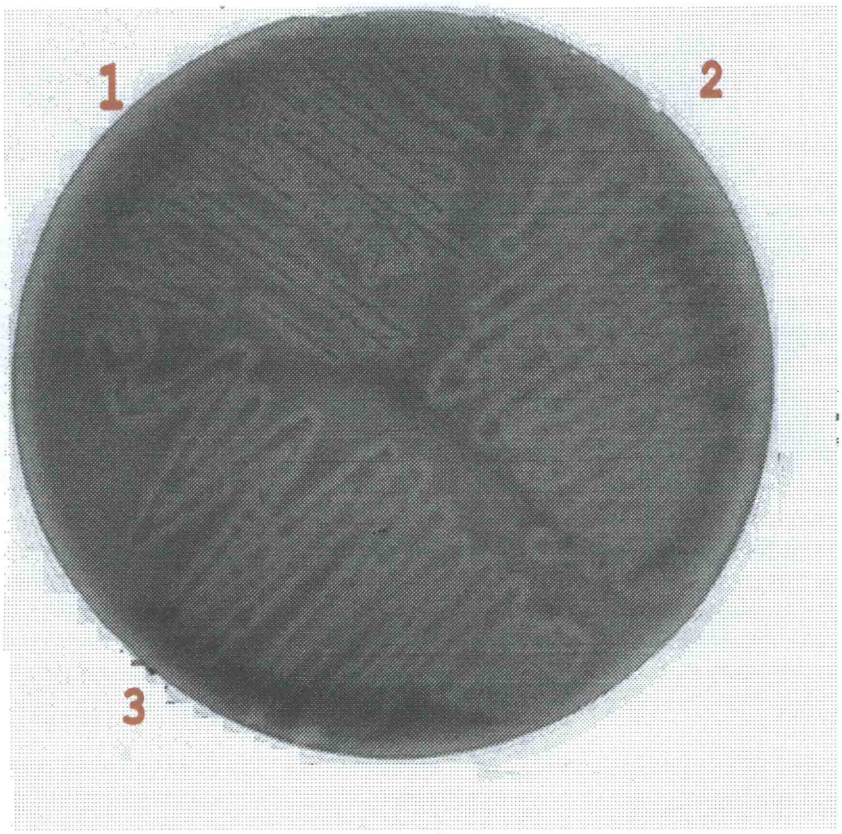


Figure 3.1.

Figure 3.2. A 1% agarose gel picture shows the products of the PCR with the genomic DNA of S43/192/4 SmR, S43/192/30R as template DNA with TT1 and TT2 as the primers. This gel was stained with ethidium bromide. The lanes are labeled in the gel.

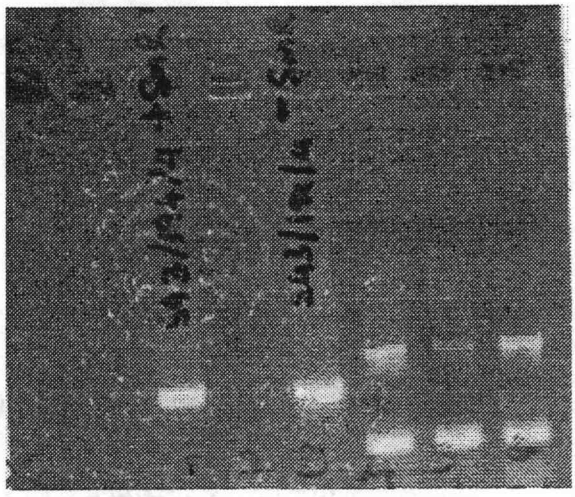


Figure 3.2.

The streptomycin resistant S43/192/4 and S43 ATCC were compared and the difference in the putative S12 gene noted, as shown in Figure 3.4. The sequences of the streptomycin resistant strains S43/192/4 and S43/192/30R were also compared and the differences between various strains are tabulated in Table 3.2. The 30R strain has a point mutation in the 56^{th} amino acid codon, changing the lysine to threonine. In the S43/192/4 SmR strain, there are two mutations when compared to the wild type *S. pyogenes*. One point mutation changed the lysine in 56 to arginine. The second mutation caused the lysine in the amino acid 101 to asparagine. The strain S43 /192/4P8 is the streptomycin resistant animal passaged S43/192/4. This strain was identical to S43/192/4 with respect to the S12 gene (Figure 3.5).

An immunoblot was done with these strains to check the production of the M protein and thus assess the potential pathogenicity of the strains. A monoclonal antibody, 10F5 was used in this blot. As this blot shows in Figure 3.6, the streptomycin resistant strains S43/192/4 and S43/192/30R have M protein in their outer cell membrane.

Figure 3.3. The S12 sequences of S43ATCC strains, both streptomycin resistant and streptomycin sensitive strains are aligned and compared. The streptomycin resistant strain was made in the lab following standard procedures. This S43 ATCC SmR strain has the lysine 56 replaced by the amino acid isoleucine. The streptomycin resistant strain is denoted as SmR and streptomycin sensitive strain as SmS.

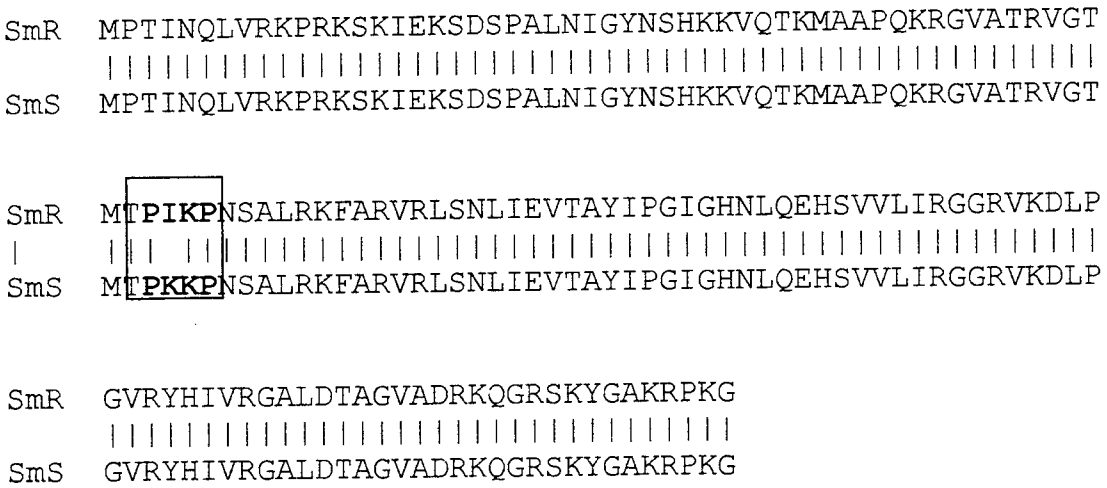

SmR MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVGT
SmS MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVGT
SmR MTPIKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVKDLP
SmS MTPKKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVKDLP
SmR GVRYHIVRGALDTAGVADRKQGRSKYGAKRPKG
SmS GVRYHIVRGALDTAGVADRKQGRSKYGAKRPKG

Figure 3.3.

Figure 3.4. The streptomycin resistant strains of *S. pyogenes,* S43/192/4 SmR and S43 ATCC SmR were analyzed. In S43 ATCC SmR strain, the lysine56 was mutated to isoleucine. The mutation of this amino acid is very frequent in S12 gene mutation in streptomycin resistance. In S43/192/4 SmR, there are two mutations, in lysine 56 and 101. The strains are referred to as ATCC and 192/4 respectively.

```
192/4 MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
ATCC  MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVG

192/4 TMTPRKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVND
      ||| | |||||||||||||||||||||||||||||||||||||||||| | |
ATCC  TMTPIKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVKD

                                                      137
192/4 LPGVRYHIVRGALDTAGVADRKQGRSKYGAKRPKG
      |||||||||||||||||||||||||||||||||
ATCC  LPGVRYHIVRGALDTAGVADRKQGRSKYGAKRPKG
                                                      137
```

Figure 3.4.

Table 3.2. Tabulation of various mutations seen in the strains of *S. pyogenes*. The streptomycin sensitivity and the mutation if any in the amino acids lysine 56 and lysine 101 are given.

Strain	Streptomycin resistance	Challenge in Mice [a]	Lysine 56	Lysine 101
S43 ATCC	Sensitive	+	Wild type (lysine)	Wild type (lysine)
S43 ATCC SmR	Resistant	+	Isoleucine	Lysine
S43/192/4	Sensitive	N.D	Lysine	Lysine
S43/192/4 SmR	Resistant	-	Arginine	Asparagine
S43/192/4 SmR P8	Resistant	-	Arginine	Asparagine
S43/192/30R	Resistant	N.D	Threonine	Lysine
T14[b]	Resistant	+	Asparagine	Lysine

a - Dr.K. Jones et al (1999) at Siga Pharmaceuticals did the mouse studies. Mice were infected intranasally with the challenge strain and the virulence of the strain in colonizing the throat, and in causing frank disease were recorded.

b - This strain T14 was studied by Tove' Bolken at Siga Pharmaceuticals. This strain is similar to S43/192/30R in properties.

Table 3.2.

Figure 3.5. Ancillary mutations in *S. typhimurium*, cause avirulence, which are quickly overcome and the second mutation is compensated by yet another mutation. But, in *S. pyogenes* strain S43/192/4, eight passages in mice have not reversed the ancillary mutation as seen in this Figure. The strain still stayed avirulent. The first sequence to be compared is S43/192/4 SmR, and the latter is the strain S43/192/4P8.

```
MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVGTMTPR
||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVGTMTPR

KPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVNDLPGVRYHIVR
||||||||||||||||||||||||||||||||||||||||||||||||||||||||
KPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVNDLPGVRYHIVR

GALDTAGVADRKQGRSKYGAKRPKG
|||||||||||||||||||||||||
GALDTAGVADRKQGRSKYGAKRPKG
```

Figure 3.5.

Figure 3.6. An immunoblot was done with S43ATCC strain as the positive control, *S. gordonii* strain as the negative control. The anti M antibody 10F5 was used and the immunoblot used alkaline phosphatase as the colormetric agent. The anti mouse antibody was tagged with the substrate. The strains used are indicated on the membrane.

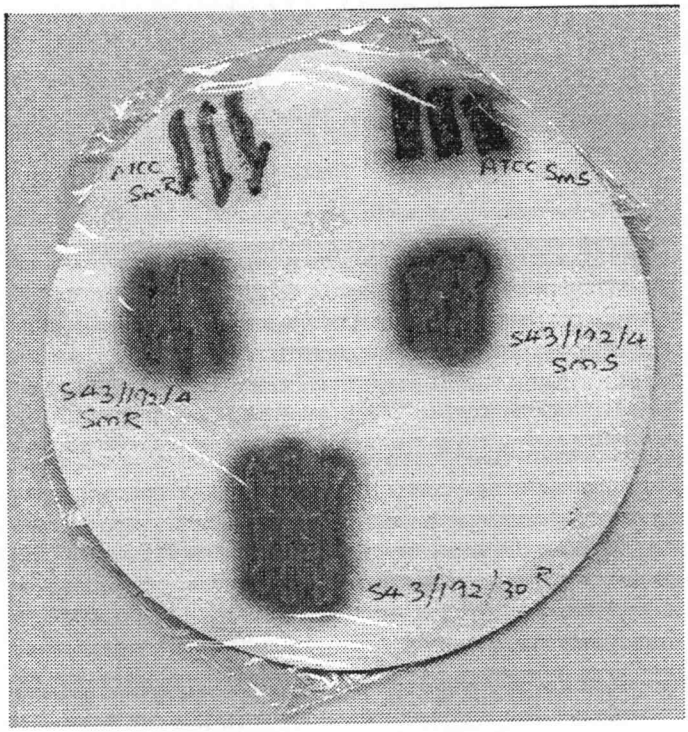


Figure 3.6.

Further, the M protein in the case of S43/192/4 was not a secreted protein, as the M protein pattern did not match that of the secreted protein.

The competition ELISA graph seen is in Figure 3.7. This graph was designed to show the % inhibition of the strains. After adjusting for the blank, the reading in each cell dilution was compared with the antibody alone reading, which was designated at 100%. Thus the compared percentages for every cell dilution of every strain were computed. The results as they were done in triplicate were averaged and the graph plotted. The graph shows that the S. pyogenes strain S43/192/4 SmR is almost similar to the strains 30R and S43ATCC in the 50% inhibition rate. The negative control showed no inhibition.

The pharyngeal adhesion assay tested for the presence of surface proteins required for the adhesion of the bacterium to the cell (Fluckiger et al., 1998), (Loimaranta et al, 1998). A competition assay was set up between the "luci" cells containing the luciferase plasmid and known to adhere to the pharyngeal cells and the strains tested. The ratio between the test strain and the luci was varied and MEM was used as the medium on

Figure 3.7. The graph of a competition ELISA is seen. The cells are increased in two fold dilutions. The data is presented as % inhibition, that is the amount of M protein present on the surface of the cell which binds to a predetermined amount of the anti M protein monoclonal antibody, 10F5 and the unbound residual antibody is tittered. If the amount of M protein is more, the level of antibody detected drops.

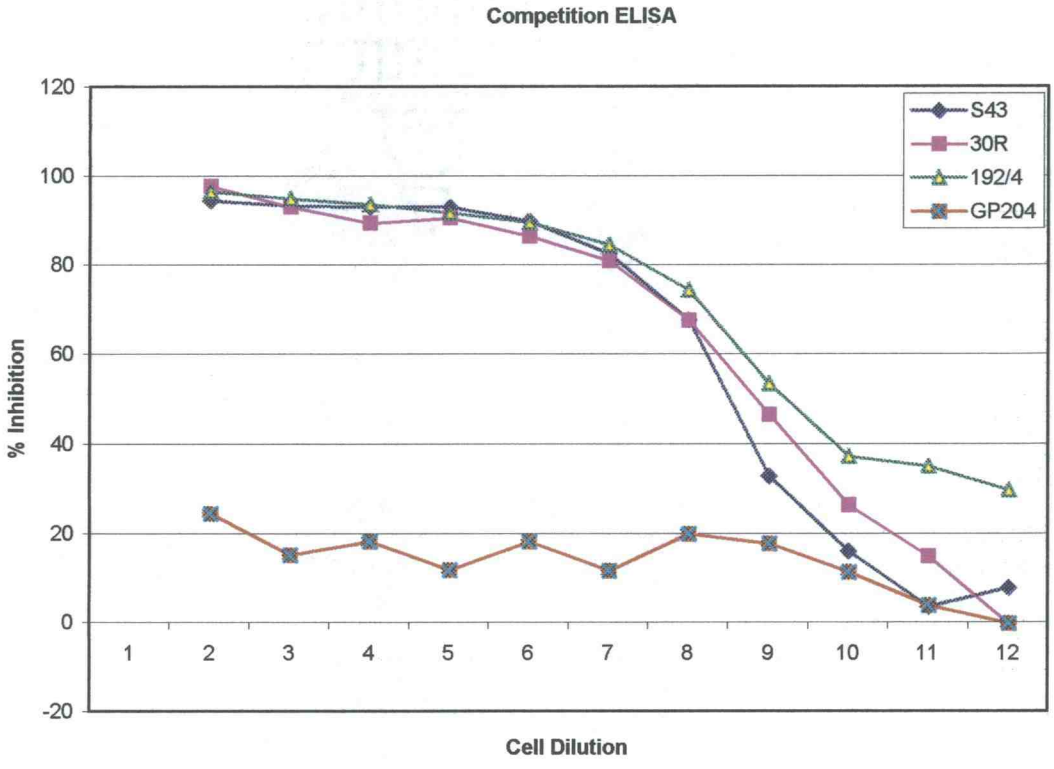

Competition ELISA
120
100
80
60
40
20
0
-20
% Inhibition
1
2
3
4
5
6
7
8
9
10
11
12
Cell Dilution
S43
30R
192/4
GP204

Figure 3.7.

Figure 3.8. The pharyngeal assay measures the competition in adhering to the pharyngeal cells by the two strains, the "luci" strain, which contains the luciferase plasmid and the test *S. pyogenes* strain. If the test strain does not adhere at all or if its adherence properties are less, then this data would show up in the luminescence units and would be equal to the luci strain alone.

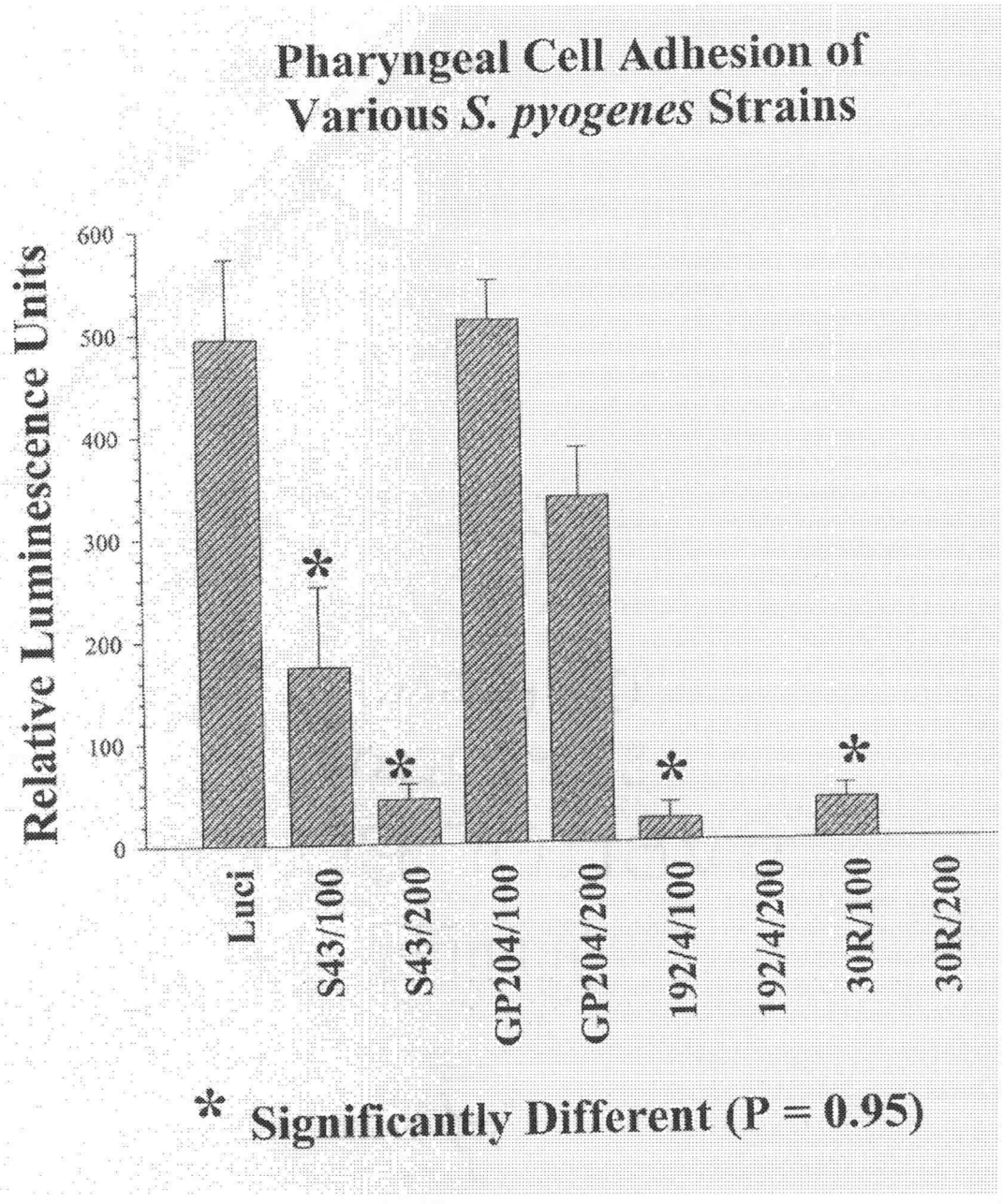
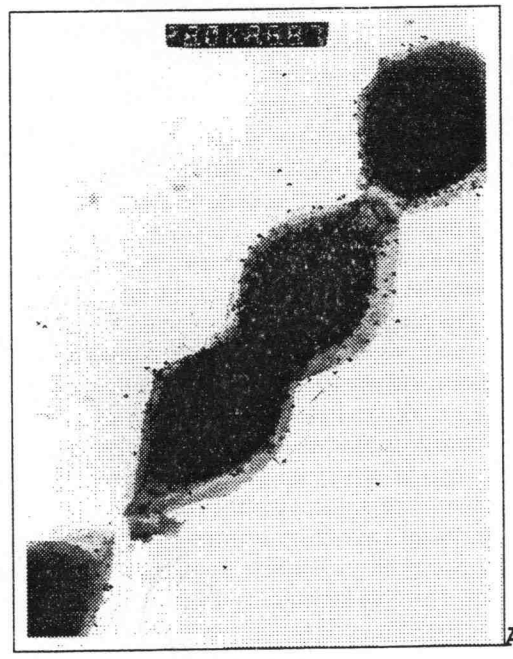

Pharyngeal Cell Adhesion of
Various S. pyogenes Strains
Relative Luminescence Units
600
500
400
300
200
100
0
Luci
S43/100
S43/200
GP204/100
GP204/200
192/4/100
192/4/200
30R/100
30R/200
* Significantly Different (P = 0.95)

Figure 3.8.

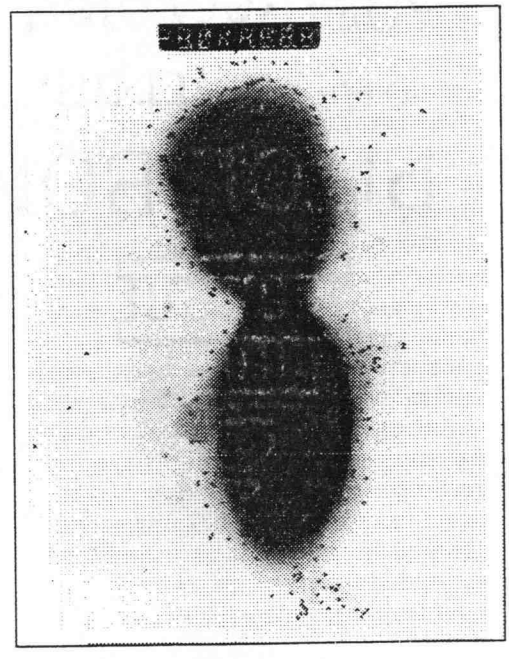
the cells. The results are depicted in the Figure 3.8. The test strains were added in 100 µl and 200 µl and the luci strain was added at a constant 100 µl per epithelial cell well. The results from this experiment show that S43/192/4 SmR and S43/192/30R strains adhere better than S43 ATCC. There is trend in the adherence of S43/192/4 SmR being lesser than S43/192/30R, but the design of this experiment did not allow for detection of subtle differences.

The electron microscopy pictures in Figure 3.9 show the presence of M protein on the surface of the *S. pyogenes* strains. This experiment looked for the presence of M protein on the surface of the bacterium, any visual properties such as secretion after a time point, less numbers in detectable range, and the interaction of the M protein and the monoclonal antibody 10F5. The gold colloidal anti mouse antibody was used to detect the monoclonal 10F5. The presence of gold indicates the presence of M protein in S43 ATCC, S43/192/4 SmR, and S43/192/30R strains.

Figure 3.9. Electron microscope pictures of the various strains of *S. pyogenes*. Goat anti mouse immunogold antibodies were used to identify the anti M monoclonal antibody 10F5, bound to the M protein on the bacterial surface. A denotes the strain S43 ATCC, B refers to the strain S43/192/30R, C refers to the streptomycin resistant strain S43/192/4 and D denotes the negative control strain *S. gordonii* GP204. The magnification of these pictures is 28K X, except for C, which has a magnification of 35K X.



A



B

Figure 3.9.

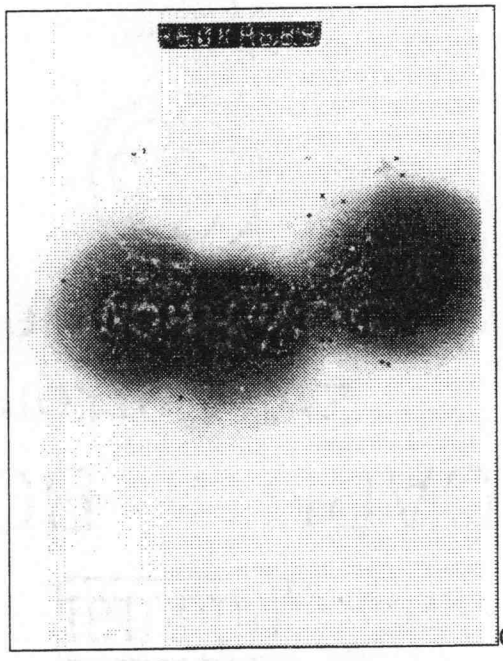


Figure 3.9.(Contd).

Discussion

Comparing the colony morphology of *S. pyogenes* strains S43/192/30R, S43 ATCC and S43/192/4, S43/192/30R and S43 ATCC colonies look similar. These colonies were mucoid in appearance and become dry and flat later. This is due to the production of the hyaluronidase enzyme and this enzyme breaks the hyaluronic acid capsule and hence the colonylooks dry. The strain S43/192/4 has white, convex and regular colonies and these colonies do not change over time. The S43/192/4 strain, hence is different in either capsule formation or in surface proteins due to this colony morphology.

Analyzing the sequences of the S12 gene of the strains of *S. pyogenes* S43 ATCC and S43 ATCC SmR, it is clear that the streptomycin resistance in the S43 ATCC strain is caused by the point mutation in the amino acid 56. The lysine 56 is mutated to isoleucine. This is unique because in most bacteria the lysine changes to threonine, arginine or asparagine, although there is a report of an str^d mutant with a change to glutamine from lysine 42 in *E.coli* (Funatsu and Wittman, 1972). This mutation to isoleucine is interesting. In the strain

S43/192/4, there are two mutations, one in lysine 56 and another in lysine 101. Lysine 56 changes to arginine and the lysine 101 to asparagine in streptomycin resistant S43/192/4. This is significant because both the lysine 56 and 101 are the two hotspots for mutation in streptomycin resistance in S12 gene in most bacteria. The mutation occurs in either one of the spots leading to streptomycin resistance but mutations in both the hotspots have not been seen to our knowledge.

The S12 gene sequence of *S. pyogenes* and *S. gordonii* V288 strain were aligned and were found to have over 90% consensus (Figure 3.10). Thus the *rpsL* gene is conserved within the family.

It was observed that the S43/192/4 strain is not pathogenic in mice (K Jones, SIGA Pharmaceuticals 1999, personal communication). This strain was used to inoculate mice with *S. pyogenes*, but the mice did not exhibit disease. In literature, it has been shown that restrictive mutations in *rpsL* in *S.typhimurium* are associated with a loss of virulence in mouse model system (Bjorkman et al., 1998). In addition, virulence-restoring compensating mutations arise with high

Figure 3.10. The S12 gene sequence of *S. pyogenes* S43ATCC strain and *S. gordonii* V288 strain are aligned and compared. As seen, the two sequences have over 90% consensus sequences. *S. pyogenes* is represented as Spy and *S. gordonii* as Sg.

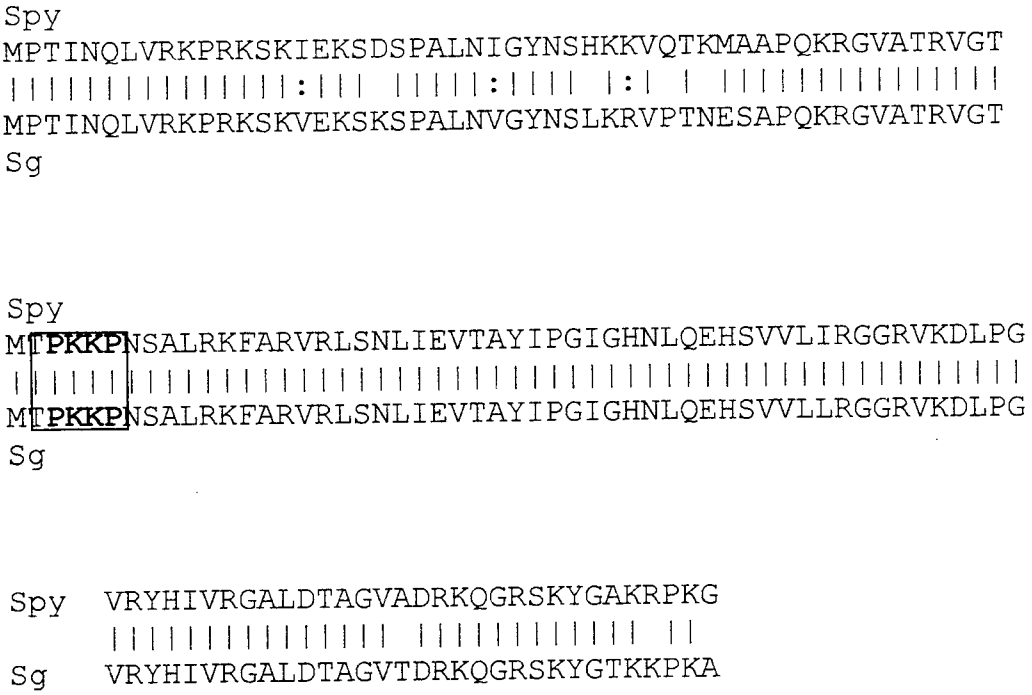

Spy
MPTINQLVRKPRKSKIEKSDSPALNIGYNSHKKVQTKMAAPQKRGVATRVGT
MPTINQLVRKPRKSKVEKSKSPALNVGYNSLKRVPTNESAPQKRGVATRVGT
Sg
Spy
MTPKKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLIRGGRVKDLPG
MTPKKPNSALRKFARVRLSNLIEVTAYIPGIGHNLQEHSVVLLRGGRVKDLPG
Sg
Spy VRYHIVRGALDTAGVADRKQGRSKYGAKRPKG
Sg VRYHIVRGALDTAGVTDRKQGRSKYGTKKPKA

Figure 3.10

frequency in the mouse, and all of this occurs in the *rpsL* gene. But in S43/192/4, eight passages in mice did not change the mutation or cause any other mutations, as seen in the S43/192/4P8. Can the double mutation in the S12 gene, the mutations in both the mutational hotspots for nonrestrictive streptomycin resistance mutations, lysine 42 and 87 in *E.coli* nomenclature, cause loss of pathogenicity? That this kind of double mutation is unique leads to this question of mutations causing stable non-virulence in *S. pyogenes*.

Secondary structure predictions done on Omiga software showed no significant change in the predicted structure following the double mutation.

To explore the cause of non-virulence in S43/192/4, the M protein of this strain was studied. The presence of M protein was confirmed with the immunoblot. The immunoblot also suggested that the M protein of S43/192/4 strain was not being preferentially secreted.

The competition ELISA graph shows that the amount of M protein in S43/192/4 was not significantly different from the amount of M protein in S43/192/30R and S43ATCC. A trend was seen in one experiment where the S43/192/4 SmR was one fold less than the other strains. This

experiment checks the M protein on the cell surface of the strains and if the M protein is less and hence more antibodies that bind the M protein were free to bind the test protein and be detected. Though the statistical significance of this observation, a single fold difference, is debatable, this is an indication that the strain S43/192/4 binds less anti M protein antibodies than S43 ATCC and S43/192/30R streptomycin resistant strains.

The pharyngeal cell adhesion assay tested for the adhesins on the bacterium to host tissue. It has long been speculated that M proteins may also act as adhesins, but the mechanisms remain unclear. Other receptors responsible for mediating streptococcal adherence include fibroblasts, fibronectin binding proteins FBP54, and SfbI/protein F (Navarre and Schneewind, 1999). The binding of M protein is speculated to occur through the C-repeat domains. This assay did not give consistent results with the *S. pyogenes* strains. But the numbers from these experiments indicate that the S43/192/4 SmR, S43/192/30R, and S43 ATCC strains all adhere to the pharyngeal cell membrane. It is evident that the S43/192/4 strain is endowed with adhesion properties and

the amount of adhesion and the differences between S43/192/4, S43ATCC and S43/192/30R should be garnered from different set of experiments.

The electron microscopy pictures suggest that the S43 ATCC, S43/192/4, and S43/192/30R all have comparable amounts of M protein on their cell surface. The M protein on the surface of S43/192/4 binds the monoclonal anti M protein antibodies. Hence that part of the M protein that binds the antibody has not been altered structurally or otherwise.

It has to be noted that all the experiments designed, the ELISA, immunoblot, and the pharyngeal assay zoned in the C repeat region of the M protein. The monoclonal antibodies used in this study, 10F5 and 20B6,bind to a region in the C repeat domain. The adhesive properties are speculated to be in the C repeat region. Hence the variation, which might be in another part of the M protein, needs to be studied further.

Ancillary mutations have been seen in *E.coli* (Timms et al., 1993). But these ancillary mutations occurred in Sm^D mutants, the strains dependant on streptomycin for growth, not in Sm^R strains in this study. These ancillary mutations in the Sm^D *E.coli* are hypothesized to give an

increased growth rate in the transition from streptomycin presence to absence, and hence increased viability. No such advantages are seen in S43/192/4, though the theory of the ancillary mutation giving an edge to the bacterium is intriguing, and would explain the stability of the ancillary mutation. The rate of occurrence of ancillary mutations in *rpsL* is many orders of magnitude higher than the expected rate (10^{-10} per base pair per replication), if these mutations are independent of the primary mutation (Timms et al., 1995) (Boe et al., 1992). The hypothesis put forward is that the mutations in the *rpsL* gene are not random, but are clustered. The *rpsL* gene region experiences periodic episodes of hypermutation. Also, the ancillary mutation may reflect the hypermutable stress state triggered by the expression of the first mutation.

In conclusion, streptomycin resistant mutants of *S. pyogenes* were studied. Mutants S43ATCC SmR and S43/192/30R were similar to other streptomycin resistant mutants of *S. gordonii* and other bacteria and had a point mutation in the S12 gene changing the lysine 56. The S43ATCC SmR strain had an isoleucine replace the lysine 56. This is a novel mutation and has not been seen

before at lysine 56. The S43/192/4 strain had two mutations, in lysine 56 and lysine 101. These two mutations are in the two hotspots of mutation in the rpsL gene. Most bacteria have a mutation in lysine 56 or in lysine 101 leading to streptomycin resistance but not in both. The experiments designed to study the M protein and the adherence properties of S43/192/4 did not show any abnormal results, though the amount of M protein in S43/192/4 seems marginally less than the other strains. Thus the non-pathogenicity of S43/192/4 SmR may be due to a posttranslational modification of the M protein, rendering it less antigenic. As the S12 protein is part of the translational apparatus, these mutations may affect more proteins and hence the pathogenicity also (Curran et al., 1995)(Fujii et al., 1999). The future study of this strain S43/192/4, can target on some known proteins of the bacterium, to decipher the cause for loss of pathogenicity. As this study proved that the M protein and adherence factors play a lesser role in S43/192/4 strain's lesser virulence, other novel ideas attributing to the avirulence should be considered and tested. This information and the work done in this study would throw light on the importance of the translational

apparatus in virulence and provide yet another method of attenuating virulence in bacteria. It is also interesting that the second mutation in S43/192/4 was not repaired or a compensatory mutation incorporated as seen in *S. typhimurium* (Bjorkman et al., 1989). This relative stability of the second mutation should also be examined. This study establishes that many different kinds of mutation can lead to streptomycin resistance. But these mutations occur in certain amino acids (42 and 87; *E. coli* nomenclature) and only in one hotspot. Thus mutation in one hotspot leads to streptomycin resistance, but mutations in both lead to further repercussions as loss of virulence. We already know that in *E. coli*, translation abnormalities occur due to streptomycin resistance (Timms et al., 1993). Hence the effect of a double mutation should be studied, in the future.

CHAPTER 4
CONCLUSION

Streptococcus pyogenes is a gram-positive coccus, which causes a variety of diseases, including rheumatic fever. As an approach to a preventative vaccine, a bacterial commensal vector expressing a conserved antigen of this pathogen uses streptomycin resistance as the antibiotic marker. Prior to entry into clinical trials, it was important to determine the nature of streptomycin resistance so that the potential safety of the vaccine strain can be assessed. Using various PCR based techniques, the *rpsL* gene of the vector *Streptococcus gordonii* was isolated and sequenced. The *rpsL* gene codes for the S12 protein of the ribosomal complex and is important in translation. Streptomycin resistance in *S. gordonii* was studied using the streptomycin resistant strains GP204, SP204(1-1) and SP635. SP635 is a vaccine strain, in which the C repeat immunogenic region of *Streptococcus pyogenes* was integrated into the chromosome. As predicted from literature, the lysine 56 of the *rpsL* gene was mutated to threonine in resistant strains. This mutation

follows a pattern seen in many bacteria. Cloning this gene and transformation into a streptomycin-sensitive recipient showed that streptomycin resistance was not a dominant trait. Presence of this resistance did not alter the growth pattern of the bacterium, nor cause any other unusual phenotypic characteristics. Hence it was concluded that the streptomycin resistance caused by the point mutation changing lys 56 to thr was likely safe to be used in vaccine strains.

Streptomycin resistance in *Streptococcus pyogenes* was studied using PCR techniques. The streptomycin resistant strains had mutations in the *rpsL* gene Two strains had lysine 56 mutated to isoleucine and threonine respectively. The mutation to isoleucine has not been previously encountered. The strain S43/192/4 SmR had two mutations, one in lysine 56 and a second in lysine 101, the two hotspots in streptomycin resistance mutation. Animal studies demonstrated that this strain was less virulent than wild type *S. pyogenes*. To ascertain if the two mutations in *rpsL* gene was the cause of loss of pathogenicity, the M protein on the surface and the adherence properties of the strain was tested. Competition ELISA showed no discernable

difference in the levels of M protein. Adherence to pharyngeal cells showed similar adhesion properties. The M protein was also detected using immunogold antibodies under the electron microscope. These also did not show any difference.

The streptomycin resistance in *S. pyogenes* is caused by mutations to the amino acid lysine 56. The strain S43/192/4 showed unusual mutation at residue 101. If the mutations in the two hotspots in the *rpsL* gene cause avirulence, this phenomenon should be studied further. It would be interesting to know this as this will provide another potential method of attenuating a virulent bacterium. The advantages offered by this mutation should also be studied as the mutation is stable and if the clustered mutation theory is correct, this mutation will shed light on this theory.

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APPENDIX

APPENDIX

CF 56
5′ CTC GTG TWG GWA CAA TGA CAC 3′

CF 57
AAC WCC WGM AGT ATC AAG YGC 3′

CF 58
5′ GMA GTA TCA AGY GCD CCA CG 3′

RV1D
5′ ACC TAA CTC TGC CCT TCG TAA A 3′

RV2D
5′ TGC TCG TGT ACG TTT GAG TAA C 3′

RV3D
TGT TGT GCT TCT TCG TGG TG 3′

RV1U
5′ CGC RCC ACG AAC GAT ATG GTA AC 3′

RV2U
5′ TAC ACG TCC ACC ACG AAG AAG C 3′

RV3U
GAT ATA AGC AGT TAC TTC G 3′

RVRSECORI
5′ NNN NNN NNN NGA ATT C 3′

RVRSBAMHI
5′ NNN NNN NNN NGG ATC C 3′

RVPRM1
5′ GAA GAT CTT CGT CAG GGT GAA TAT TAC TAA 3′

RVPRM2
5′ AAC TGC AGG TTA TCG ATT TTT AAT TAT T 3′

RVPTRKPSTI
5′ AAC TGC AGA TGC CTA CAA TTA ACC AAT TGG T 3′

RVPTRKBAMHI
5′ CGG GAT CCC GTT ATG CTT TTG GTT TTT TAG T 3′
TT1
5′ CAC CAA GTG CTA TAT CAA CC 3′

TT2
5′ CTC TTA TCC CCT TCT ATT ATC C 3′

RVRPSL5′
5′ CGG GGT ACC GAT GCC TAC AAT TAA CCT GGT TA 3′

RVRPSL3′
5′ CCC AAG CTT TTA TTA TGC TTT TGG TTT TTT AG 3′